AD-A046 319 OREGON UNIV HEALTH SCIENCES CENTER PORTLAND DEPT OF --ETC F/G 6/13

PHYSIOLOGY AND METABOLISM OF 'NEISSERIA GONORRHOEAE'.(U)

DEC 76 S A MORSE DAMD17-74-C-4139

UNCLASSIFIED NL

1 OF 1

AD A046319

END

DATE FILMED

12-77

DDC

PHYSIOLOGY AND METABOLISM OF *NEISSERIA GONORRHOEAE*

FINAL REPORT

STEPHEN A. MORSE, PH.D.
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
UNIVERSITY OF OREGON HEALTH SCIENCES CENTER

DECEMBER, 1976

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D.C. 20314

Contract No. DAMD17-74-C-4139

University of Oregon Health Sciences Center
Portland, Oregon 97201

9 Final rept. 1 Jul 74-30 Jun 76,

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) 6 Physiology and Metabolism of Neisseria gonorrhoeae.		5. TYPE OF REPORT & PERIOD COVERED Final Report July 1, 1974 to June 30, 1976
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) 10 Stephen A. Morse ~~PhD.~~		8. CONTRACT OR GRANT NUMBER(s) 15 DAMD17-74-C-4139 new
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Microbiology & Immunology University of Oregon Health Sciences Center 3181 SW Sam Jackson Pk. Rd., Portland, OR 97201		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS ~~61102A~~ 16 17 3A161102B71Q 00 ~~030~~
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command, Washington, D.C. 20314		12. REPORT DATE 11 Dec[redacted]76
		13. NUMBER OF PAGES ~~50~~ 12 49p.
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for Public Release; Distribution Unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Neisseria gonorrhoeae: Electron transport chain, Cytochromes, tricarboxylic acid cycle, enzymes, growth, glucose metabolism, identification.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report consists of studies which have examined various aspects of the physiology and metabolism of Neisseria gonorrhoeae. The principal findings have been:

(1) The respiratory chain appears to contain several nonheme iron centers, cytochrome c, two b cytochromes, with cytochrome o which probably serves as the terminal oxidase.

(OVER)

New

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

410 462

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. (2) Acetate accumulates during glucose catabolism and is not further metabolized until glucose catabolism ceases. Then, acetate is oxidized by the tricarboxylic acid cycle (TCA cycle).

(3) Although acetate does not enter the TCA cycle during glucose catabolism, a portion of the cycle functions as evidenced by the oxidation of glutamate.

(4) The gonococcus does not contain a pyridine nucleotide-dependent malate dehydrogenase. Instead, this organism possesses a FAD-dependent malate oxidase which appears to be associated with the electron transport chain.

(5) A rapid method was developed to identify Neisseria spp. by the production of acid from specific carbohydrates.

AD ____________

PHYSIOLOGY AND METABOLISM OF NEISSERIA GONORRHOEAE

FINAL REPORT

STEPHEN A. MORSE, PH.D.
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
UNIVERSITY OF OREGON HEALTH SCIENCES CENTER

DECEMBER, 1976

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D.C. 20314

Contract No. DAMD17-74-C-4139

University of Oregon Health Sciences Center
Portland, Oregon 97201

SUMMARY

This Final Report consists of studies which have examined various aspects of the physiology and metabolism of Neisseria gonorrhoeae. The primary objectives of this research were to:

a) Define the nutritional factors in the environment which determine or modify the virulence and/or antigenic composition of N. gonorrhoeae.

b) Delineate the role of the physical environment in determining the virulence and antigenic composition of N. gonorrhoeae.

c) Develop better methods for growth, identification and maintenance of the virulence of N. gonorrhoeae through an understanding of its nutrition and physiology.

d) Ascertain whether differences exist in the metabolism, physiology, and virulence of strains isolated from disseminated and non-disseminated gonococcal infections.

e) Obtain an antigenic fraction which is specific for N. gonorrhoeae under all conditions of growth for use as a tool in the diagnosis of gonorrheal infections and as a potential immunogen.

The results of studies presented herein may be summarized as follows: The cell membrane-associated respiratory electron transport chain of Neisseria gonorrhoeae was examined using electron paramegnetic spectroscopy (EPR) at liquid helium temperatures and optical spectroscopy at liquid nitrogen and room temperatures. EPR spectra of dithionite reduced particles indicated the presence of centers N-1 and N-3 in the site I region of the respiratory chain, while reduction with succinate revealed the existence of center S-1 from the succinate cytochrome c reducatase segment. Free radical(s) resembling that due to flavin semiquinone were observed with both reductants. Low temperature (77 K) optical difference spectra indicated the presence of cytochromes with alpha band maxima at 549, 557, and 562. Bands at 567, 535, and 417 nm, characteristic of the CO compound of cytochrome o were also identified. Cytochromes a_1 and a_3 were not detected; however a broad but weak absorbance with an alpha band maximum at 600 nm and a Soret shoulder at 440 nm was observed. Hence the respiratory chain of N. gonorrhoeae appears to contain several non-heme iron centers, cytochrome c, two b cytochromes, with cytochrome o which probably serves as the terminal oxidase.

Tricarboxylic acid cycle activity was examined in Neisseria gonorrhoeae strain CS-7. The catabolism of glucose in N. gonorrhoeae by a combination of the Entner-Doudoroff and pentose phosphate pathways results in the accumulation of acetate which was not further catabolized until the glucose was depleted or growth became limiting. Radiorespirometric studies revealed that the label in the 1 position of acetate was converted to CO_2 at twice the rate of the label in the 2 position indicating the presence of a tricarboxylic acid cycle. Growth on glucose markedly reduced the levels of all tricarboxylic acid cycle enzymes with the exception of citrate synthase (EC 4.1.3.7). Extracts of glucose-grown cells contained detectable levels of all tricarboxylic acid cycle enzymes except aconitase (EC 4.2.1.3), isocitrate dehydrogenase (EC 1.1.1.42), and a pyridine nucleotide-dependent malate dehydrogenase (EC 1.1.1.37). Extracts of cells capable of oxidizing acetate lacked only the pyridine nucleotide-dependent malate dehydrogenase. In lieu of this enzyme a particulate pyridine nucleotide-independent malate oxidase (EC 1.1.3.3) was present. This

enzyme required flavin adenine dinucleotide for activity and appeared to be associated with the electron transport chain. Radiorespirometric studies utilizing labeled glutamate demonstrated that a portion of the tricarboxylic acid cycle functioned during glucose catabolism. In spite of the presence of all tricarboxylic acid cycle enzymes, N. gonorrhoeae CS-7 was unable to grow in medium supplemented with cycle intermediates.

A suspending medium was developed for use with the Minitek system for the confirmatory identification of Neisseria gonorrhoeae, N. meningitidis, and N. lactamica based upon the production of acid from various carbohydrates. The addition of sodium bicarbonate to the medium made negative reactions easier to read. More isolates of N. gonorrhoeae were identified using the suspending medium in the Minitek system than using CTA media. With a suitable inoculum size a positive identification could be made in less than 1 hour; most isolates (90.8%) could be identified within 4 hours of inoculation. The Minitek system is reliable and easy to use.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

PARTIAL CHARACTERIZATION OF THE RESPIRATORY CHAIN OF NEISSERIA GONORRHOEAE.

A. Introduction.

Neisseria gonorrhoeae is an obligate aerobe whose only natural habitat is man (1). This organism possesses a terminal oxidase with a high specific activity which catalyzes the oxidation of tetramethyl-p-phenylenediamine (TMPD), a property shared with other members of this genus and most aerobic bacteria. Other than this terminal oxidase reaction (2) little is known about the electron transport components of the organism. Jurtshuk and Milligan (3) have reported difference spectra of the cytochromes observed in N. catarrhalis but no information on the site I region of the respiratory chain was presented. Furthermore, N. catarrhalis has been shown to be genetically dissimilar from the true Neisseria (4) and has subsequently been reclassified Branhamella catarrhalis (1). Hence many of the respiratory components of the true Neisseria have not been previously reported.

This study reports steady-state data on substrate and dithionite reducible components associated with the membrane bound respiratory chain of N. gonorrhoeae. The purpose of this study is to describe and characterize some of these respiratory components and to compare them with those of other aerobic organisms as well as other species of Neisseria. Furthermore, with the wealth of information concerning the reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase region of many eucaryotic cells (5,6), it appeared timely to examine the low temperature (<77 K) electron paramagnetic resonance (EPR) spectra of N. gonorrhoeae to establish whether similarities exist between this organism and its host.

B. Materials and Methods.

Organism. Neisseria gonorrhoeae strain CS-7 was used in this investigation. The properties and maintenance of this strain have been previously described (7,8) as have the medium and cultural conditions (9).

Preparation of cell membranes. Stationary-phase cultures were harvested by centrifugation (16,000 x g for 10 min) in a refrigerated centrifuge, washed twice and resuspended in 0.4 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.4) containing 0.005 M beta-mercaptoethanol. The cell suspension was disrupted by sonication (Biosonik IV; Bronwill, Rochester, N.Y.) for a total of 3 min in 30 sec pulses (40 percent maximum output) with 1 min intervals for cooling. The resulting suspension was centrifuged at low speed (23,000 x g for 15 min) to remove cell walls and unbroken cells. The supernatant was centrifuged at 100,000 x g for 2 hr (type 30 rotor, Spinco model L5-65 ultracentrifuge) and the membrane-rich pellet was resuspended in a small volume of 0.4 M Tris-HCl buffer (pH 7.4) containing 0.005 M beta-mercaptoethanol and stored at -30 C until used.

Visible spectroscopy. Room temperature and liquid nitrogen (77 K) spectra of the membrane-rich fraction were recorded on a Cary Model 14 spectrophotometer equipped with a model 1462 scattered transmission attachment. The spectrophotometer was calibrated with a holmium oxide standard immediately prior to use. Samples to which reducing agents were added were first made anaerobic by alternately evacuating and flushing with argon. Liquid nitrogen difference spectra were recorded in a 0.2 cm path length cuvette attached to a low temperature tee (10). This tee was then partially immersed in liquid nitrogen contained in a glass Dewar fitted with quartz windows.

Carbon monoxide difference spectra were obtained by gently bubbling CO gas through anaerobic dithionite reduced samples. Samples were periodically withdrawn for spectroscopic analysis.

EPR spectroscopy. Membrane-rich samples (50 mg protein/ml) were thawed and transferred to anaerobic EPR tubes. Samples were evacuated and flushed with argon at least 8 times, reduced with solid sodium dithionite or succinate and frozen in liquid nitrogen. Liquid helium spectra were recorded with a Varian V-4502 EPR spectrometer. The samples were cooled with a liquid helium transfer system (Model LTD-3-110, Air Products and Chemicals, Inc., Allentown, Pa.). Temperatures were monitored with a germanium resistor.

Miscellaneous measurements. Protein was determined by the method of Lowry *et al.* (11) using bovine serum albumin as standard.

Chemicals. Carbon monoxide and ultra high purity argon gases were obtained from Metheson Gas Products (Newark, Calif.). Sodium L-malate and L(+)lactic acid were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other reagents were analytical grade or the highest purity available.

C. Results and Discussion.

EPR spectra. Orme-Johnson *et al.* (6) and Ohnishi (5) have recently reviewed the iron-sulfur centers arising from the site I region of the respiratory chain in a variety of eucaryotic organisms. These authors have reported g-values for a number of prominent peaks detectable at the temperature of liquid helium, and have related these EPR signals to iron-sulfur centers by titrating the signals with reducing equivalents. We have examined the membrane-associated respiratory chain of the aerobic procaryote, *N. gonorrhoeae*, with low temperature EPR. Figure 1 illustrates the dithionite and succinate reduced EPR spectra of the membrane-rich fraction of *N. gonorrhoeae*. The general line shape of the dithionite reduced spectra is very similar to that of *Candida albicans* and broadly resembles the spectra from bovine heart submitochondrial particles (5). The apparent g-values of several prominent peaks are given in this figure for comparison with site I EPR spectra from other organisms.

It is evident that *N. gonorrhoeae* shows prominent signals at g = 2.02, 1.94 and 1.93 corresponding to center 1 signals according to the designation of Orme-Johnson *et al.* (6). These investigators give g-values for this center comprised of peaks designated q, r, s at g = 2.022, 1.938 and 1.923

respectively. Similarly we observed signals at g = 2.09, 1.89 and 1.87 corresponding to peaks *l, m, and n*, with g-values of 2.099, 1.886 and 1.862 respectively (center 3) (6). These signals, corresponding to centers N-1 and N-3, would be observed upon reduction with dithionite if the site I respiratory chain region of N. gonorrhoeae has components similar to those of mammalian mitochondria. We did not observe signals with field positions corresponding to center N-2 at potentials attained with excess succinate or dithionite. Evidence for all the EPR active centers present in N. gonorrhoeae membrane will require quantitative redox titration. It should be noted that slightly different g-values for site I centers were reported by Ohnishi (5) for C. utilis.

Both the dithionite and succinate reduced samples have a g = 2.00 free radical sign, probably arising from a flavin radical as is the case in mitochondria. The succinate reduced spectrum displays a g = 1.94 central resonance similar to center S-1 observed in succinate dehydrogenase by Beinert and Sands (12). The low potential center S-2 (13) was not observed in gonococcal membrane fractions due to overlap with site I EPR signals. The high potential iron sulfur protein signals recently observed in the succinate CoQ reductase region (14) were not examined.

Optical difference spectroscopy. Low temperature (77 K) reduced-minus-oxidized spectra of N. gonorrhoeae cell membrane fragments are shown in Figure 2. Three alpha bands are resolved in the dithionite reduced-minus ferricyanide oxidized spectra with absorption maxima at 549 (c-type cytochrome) and 557 with a shoulder at 562. The latter two, together with the beta maxima at 528 nm are indicative of b-type cytochromes (15). In addition, there is a broad absorption band at 600 nm. The increased absorbance observed upon addition of ferricyanide to the reference cuvette is the result of a more complete oxidation of the respiratory chain components, especially those which are not autoxidizable. This was especially apparent in this case since the buffer in which the gonococcal membranes were suspended contained β-mercaptoethanol which can donate reducing equivalents to the respiratory chain, necessitating the use of a more effective oxidant. Room temperature difference spectra of dithionite reduced-minus ferricyanide oxidized (not shown) contain absorbance maxima in the alpha region centered at 553, 560, and 600 nm. These bands were approximately one-fifth as intense at room temperature than at 77 K. The 25 C beta absorbances are centered at 516, 523, and 530. Low temperature dithionite reduced-minus air oxidized difference spectra (Fig. 2) gives a Soret maximum at 427 nm. This peak is shifted about 4 nm to lower wave length when the reducing agent is ascorbate. In either case, the Soret band is unresolved but displays a shoulder at 440 nm, typical for a-type cytochromes (15), and a trough at 450-452 nm probably due to flavin in various states of reduction.

The observation of a broad absorption band centered around 600 nm with a 440 nm Soret shoulder may indicate the presence of an a-type cytochrome in this organism. However, the 600 nm peak does not display the normal sharpness for an alpha band absorbance of an a-type cytochrome. Furthermore, the low 440 nm extinction would indicate a very small concentration of this component. On the basis of a similar broad 600 nm absorption, Jurtshuk and Milligan (3) have suggested B. catarrhalis contained cytochrome a_1. Even though our CO difference spectra did not indicate the presence of cytochromes a_1 or a_3 (see below), we were unable to eliminate the possibility that an a-type exists in the membrane of N. gonorrhoeae.

Approximate concentrations of the various cytochromes computed from low temperature dithionite-minus-ferricyanide difference spectra by the method of Dietrich and Biggins (16) are presented in Table 1. An estimated millimolar extinction coefficient of $\sum = 20\ mM^{-1}cm^{-1}$ (16) was used together with a low temperature intensification factor of five (10) to compute these concentrations. No correction was made for band overlap. Cytochrome o was computed from the dithionite +CO minus dithionite spectrum using the estimation procedure of Daniel (17) i.e., peak minus trough $\sum = 170\ mM^{-1}cm^{-1}$). The concentrations of cytochromes c and b calculated in the above manner are higher in N. gonorrhoeae than those reported by Smith (18) for Keilin-Hartree heart muscle and other selected bacterial membranes (16,19), while cytochromes o and a-type are slightly lower in the organism described here.

Ascorbate, succinate, and L-lactate, but not L-malate, were found by optical criteria to reduce membrane-bound respiratory chain components of N. gonorrhoeae. Difference spectra of ascorbate- and succinate-reduced particles (Figure 3) contain a broad alpha region absorption centered at 551 nm and a broad weak 600 nm band. Particles reduced with succinate display a shoulder at 558-560 nm. Since ascorbate does not normally reduce b-type cytochromes, the 558-560 nm shoulder is not observed when this reductant is employed. The breadth of the absorption at 551 nm suggests that it is composed of more than one band. Reduction with L-lactate diminishes the lower wave length component to a slightly greater extent, splitting the band into 549 and 553 nm peaks.

Figure 4 illustrates that N. gonorrhoeae also contains at least one CO binding pigment. Room temperature dithionite + CO minus dithionite difference spectra shows maxima at 567, 535 and 417 nm and troughs at 553 and 438 nm. These peak and trough positions which have been attributed to cytochrome o are within the range of CO binding chromophores found in other microorganisms (15). Broberg and Smith (20) demonstrated a technique for detecting both cytochrome a_3 and cytochrome o in bacterial cell membranes. These authors found that initial bubbling with CO produced a small peak of 590 and a trough at 445 nm with a peak plus shoulder at 413 and 430 nm attributed to cytochrome a_3. Further reaction with CO produced cytochrome o absorbances similar to those described above. These differences have been attributed to different CO binding rates of these cytochromes (15). We were unable to detect absorbances due to cytochrome a_3 or cytochrome a_1 (15) by the above procedure. However, a cytochrome o absorption spectrum was detected upon bubbling dithionite reduced N. gonorrhoeae membranes for 90 sec with CO. Full development of this spectrum took 15 minutes, at which time a concentration of 0.18 nmoles cytochrome o per mg of cell membranes was computed from peak 417 minus trough 438 nm absorbances. Jurtshuk and Milligan (3) have presented a CO difference spectra from N. flava in which a typical cytochrome o spectra was also observed. However, even though the organism studied here is from the same genus, the peak and trough maxima reported by the above authors (3) are shifted three to five nm to a lower wave length. Discrepancies in peak position of this magnitude are not likely to be due to spectrophotometer calibration differences hence the equal peak position within a homogeneous genus rule (19) may not apply here.

The carbon monoxide difference spectra suggests the presence of an autoxidizable cytochrome (cytochrome o) associated with the membrane of N. gonorrhoeae. This cytochrome may be responsible for the high TMPD oxidase activity associated with members of the genus Neisseria. Even though cytochrome a_3 or a_1 could not be detected by CO difference spectra by the procedures employed here, the question of the presence of another autoxidizable cytochrome in membranes of N. gonorrhoeae is still open since its spectra may be masked by the CO compound of cytochrome o.

TRICARBOXYLIC ACID CYCLE ACTIVITY IN NEISSERIA GONORRHOEAE.

A. Introduction.

Members of the genus Neisseria are unable to utilize many carbohydrates (1). Glucose, a key intermediate in the synthesis of many important cellular components, is the only carbohydrate that can be utilized as an energy source by N. gonorrhoeae. Recent radiorespirometric studies by Morse et al. (8) have shown that growing cells of N. gonorrhoeae utilize glucose by strictly aerobic mechanisms involving the Entner-Doudoroff and pentose phosphate pathways. Acetate was the only detectable nongaseous end product produced from glucose. After the depletion of glucose, the label in the 1 position of acetate was converted to CO_2 at twice the rate of the label in the 2 position. This pattern of acetate oxidation is indicative of a functioning tricarboxylic cycle. These results were contrary to those of Tonhazy and Pelczar (21) who utilized resting cell suspensions of glucose-grown N. gonorrhoeae and concluded that of the members of the conventional tricarboxylic acid cycle, only α-ketoglutarate, succinate, fumarate, malate, and oxaloacetate could be oxidized. Resting cell suspensions were incapable of oxidizing acetate, cis-aconitate, citrate, and isocitrate. Holten and Jyssum (22) were unable to detect activity corresponding to malate dehydrogenase (EC 1.1.1.37) in cell extracts of N. gonorrhoeae, further suggesting the absence of a functional tricarboxylic acid cycle.

Direct evidence (i.e., presence of all necessary enzymes) for a tricarboxylic acid cycle in N. gonorrhoeae is lacking. This study presents evidence that a functional tricarboxylic acid cycle exists in N. gonorrhoeae and that its activity is markedly influenced by the presence of glucose.

B. Materials and Methods

Organism. N. gonorrhoeae strain CS-7 was used in these studies. This strain was isolated from the urethral exudate of a male with gonococcal urethritis. A stable T-4 colony type (59) was selected on GC agar (Difco) plates following passage in the yolk sac and allantoic cavity of a chicken embryo. The maintenance and the diagnostic criteria for identification of N. gonorrhoeae were described previously (7).

Medium. The basal medium contained the following per liter: proteose peptone no. 3 (Difco), 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 5 g; and soluble starch, 1 g. The final pH of the medium was 7.2. A growth factor supplement, identical in composition to IsoVitalex enrichment (BBL), $NaHCO_3$ (42 mg/liter), and glucose (5 g/liter) was added after autoclaving.

Growth conditions. Frozen stock cultures were prepared as described by La Scolea and Young (23). For use, the cultures were thawed at room temperature and inoculated into the basal medium. A suspension of cells from an overnight culture was used to inoculate fresh basal medium to an initial turbidity of 5 to 10 Klett units (Klett-Summerson colorimeter, filter No. 54 [540 nm]; 12-24 μg dry wt/ml). All liquid cultures were incubated at 37 C in a Model G-76 gyratory water bath shaker (New Brunswick Scientific, N.J.). Under these conditions, the generation time of strain CS-7 was approximately 70 min.

Abbreviations and chemicals. The following abbreviations were used: nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), phosphoenol pyruvate (PEP), coenzyme A (CoA), adenosine diphosphate (ADP), ethylenediaminetetraacetic acid (EDTA). All reagents and enzymes were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Radioisotopes. The following radioisotopes were obtained from the New England Nuclear Corp. (Boston, Mass.): DL-(1-^{14}C)-glutamic acid (specific activity, 51.01 mCi/mmol); DL-(5-^{14}C)-glutamic acid (specific activity, 3.69 mCi/mmol); (U-^{14}C)-arginine (specific activity, 279 mCi/mmol); (1-^{14}C)-glucose (specific activity, 80 mCi/mmol); sodium (1-^{14}C)-acetate (specific activity, 22 mCi/mmol); sodium (2-^{14}C)-acetate (specific activity, 2.0 mCi/mmol); sodium (1-^{14}C)-α-ketoglutarate (specific activity, 60 mCi/mmol); and (1,4-^{14}C)-succinic acid (specific activity, 35 mCi/mmol).

Radiorespirometry. Radiorespirometric procedures were performed as previously described (8). Cultures were harvested during the exponential phase of growth (75-85 Klett units), centrifuged (27,000 x g for 10 min) and resuspended in fresh basal medium to a final concentration of 0.8 to 0.9 mg (dry weight) per ml. Twenty-five ml of the cell suspension were added to each radiorespirometer vessel. The vessels were described previously (24) and consisted of 125-ml Erlenmeyer flasks with sidearms. Unlabeled substrates and specifically labeled substrates were added to the sidearms in a final volume of 1.0 ml. Where indicated, the final concentrations were: monosodium glutamate, 50 μmol; sodium acetate, 100 μmol; and glucose, 69.4 μmol. In some experiments unlabeled glucose (173 μmol) was added to the basal medium in order to prevent an increase of tricarboxylic acid cycle activity during equilibration. The vessels were equilibrated for 10 min at 37 C in a gyratory water bath shaker prior to the addition of substrate and radioisotope. At zero time the material in the sidearms was tipped in and [^{14}C]CO_2 collection began. The flasks were continually sparged with air at a flow rate of 65 ml per min. The collection and processing of samples and inventory of ^{14}C in these experiments has been described previously (8). The total recoveries were all within acceptable limits (92-101%).

Cell extracts. Cell extracts of glucose-grown cells were prepared from cultures grown in liquid medium containing 0.5% glucose. Cells derepressed for tricarboxylic acid cycle activity (glucose-depleted) were prepared from cultures initially grown to late log phase in liquid medium containing 0.5% glucose. These cells were harvested (16,000 x g, 10 min), resuspended in fresh medium containing 0.5% sodium acetate and incubated at 37 C for 60 min. All cultures were harvested at the end of the exponential growth phase by

centrifugation (16,000 x g for 10 min). Cells were washed twice and resuspended in 0.4 M Tris (hydroxymethyl)-aminomethane-hydrochloride buffer (pH 7.4) (Tris-HCl) containing 0.005 M β-mercaptoethanol. The cell suspension was disrupted by sonic treatment (Biosonik IV; Bronwill, Rochester, New York) for a total of 3 min in 30-S pulses (40% maximum output with 1-min intervals for cooling). The resulting suspension was centrifuged at low speed (23,000 x g, for 15 min) to remove cell walls and unbroken cells. The supernatant fluid was centrifuged at 100,000 x g for 1 hr (type 30 rotor, Spinco model L5-65 ultracentrifuge). The supernatt was split into two aliquots. One aliquot was stored at -30 C and the other was placed in ice and assayed immediately for enzymatic activity. The membrane-rich pellet was resuspended in a small volume of 0.4 M Tris-HCl buffer pH 7.4 containing 0.005 M beta-mercaptoethanol and stored at -30 C until assayed for enzymatic activity. Storage at -30 C had no appreciable effect upon enzyme activity. Prior to use, material in the pellet was sedimented by ultracentrifugation (100,000 x g for 1 hr) and resuspended in 0.4 M Tris-HCl buffer (pH 7.4).

Enzyme assays. All extracts were assayed for enzymatic activity at 25 C in a Beckman Model 25 spectrophotometer. Citrate synthase (citrate oxaloacetate-lyase [CoA acetylating], EC 4.1.3.7) was measured spectrophotometrically by the method of Parvin (25). The method described by Fansler and Lowenstein (26) was used to determine the activity of aconitase (citrate [isocitrate] hydrolyase, EC 4.2.1.3). Isocitrate dehydrogenase (threo-D_s-isocitrate:NADP oxidoreductase [decarboxylating], EC 1.1.1.42) was measured spectrophotometrically by following the increase in absorbance due to the reduction of NADP at 340 nm. The reaction mixture contained: 1.0 ml of 33 mM Tris-HCl buffer (pH 7.4), 0.1 ml of 1 mM $MnCl_2$; 0.1 ml of 2 mM trisodium-D_s-isocitrate; 0.1 ml of 1 mM KCN neutralized with 1 M HCl; 0.1 ml of 3.4 mM NADP; H_2O and cell extract (0.6-1.25 mg protein) to a final volume of 3.0 ml. The activity of the α-ketoglutarate dehydrogenase complex was determined according to the procedure of Reed and Mukherjee (27). Succinyl CoA synthetase (succinate:CoA ligase [ADP', EC 6.2.1.5) activity was measured by following the formation of hydroxamate as described by Gibson et al. (28). Membrane-bound succinate dehydrogenase (succinate:[acceptor]oxidoreductase, EC 1.3.99.1) was determined by the procedure of Johnston and Gotschlich (29). The method of Hill and Bradshaw (30) was used to measure fumarase activity (l-malate hydro-lyase, EC 4.2.1.2). Membrane-bound l-malate oxidase (EC 1.1.3.3) was determined by the spectrophotometric procedure of Jurtshuk et al. (31). Malate dehydrogenase (l-malate:NAD oxidoreductase, EC 1.1.1.37) was measured spectrophotometrically by following the decrease in absorbance due to the oxidation of NADH at 34o nm. The reaction mixture contained: 0.3 ml of 250 mM potassium phosphate buffer (pH 7.4); 0.15 ml of 1.5 mM NADH; 0.15 ml of 7.5 mM oxaloacetate adjusted to pH 7.4; H_2O and cell extract (0.6-1.25 mg protein) to a final volume of 3.0 ml. The reaction was started by the addition of oxaloacetate.

The enzymes involved in anaplerotic reactions, PEP carboxylase (orthophosphate:oxaloacetate carboxy-lyase [phosphorylating], EC 4.1.1.31) and malic enzyme (l-malate:NADP oxidoreductase [decarboxylating], EC 1.1.1.40), were determined according to the methods of Maeba and Sanwal (32) and Hsu and Lardy (33) respectively. NAD-dependent glutamate dehydrogenase (L-glutamate:NAD oxidoreductase [deaminating], EC 1.4.1.2) and NADP-dependent glutamate dehydrogenase (L-glutamate:NADP oxidoreductase deaminating, EC 1.4.1.4) were determined by the procedure of Jyssum and Borchegrevink (34).

Aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) was assayed spectrophotometrically in an assay mixture consisting of 2 ml of 50 mM triethanolamine hydrochloride buffer (pH 7.4) containing 10 mM $MgSO_4$ and 5 mM disodium EDTA; 20 μl of 100 mM potassium α-ketoglutarate (pH 6.5); 20 μl of 3 mM NADH; 100 μl of cell extract (0.6-1.25 mg protein); 100 μl of 15 mM KCN (pH 7.0); 100 μl of 100 mM pyridoxal phosphate; and 245 μl of H_2O. The reaction was initiated by the addition of 5 μl of malate dehydrogenase (10 units) and the decrease in absorbance at 340 nm recorded. Then, 10 μl of 100 mM L-aspartate (pH 6.5) was added to the cuvette and the decrease in absorbance at 340 nm followed again. The activity of the enzyme was calculated from the difference in the rates of decrease in the presence and absence of L-aspartate. Alanine aminotransferase (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) was assayed in a similar manner except that 5 μl of lactate dehydrogenase (47 units) and 10 μl of 100 mM L-alanine (pH 6.5) were added.

The glyoxylate cycle enzymes, malate synthetase (L-malate glyoxylate-lyase [CoA acetylating], EC 4.1.3.2) and isocitrate lyase (threo-D_s-isocitrate glyoxylate lyase, EC 4.1.3.1), were measured according to the procedures of Dixon and Kornberg (35) and Carpenter and Beevers (36) respectively. Citrate lyase (citrate oxaloacetate lyase, EC 4.1.3.6) was measured by the procedure of Dagley (37).

All enzyme reactions were linear with respect to time.

Visible spectroscopy. Liquid nitrogen (77 K) spectra of the membrane-rich fractions from glucose-grown and glucose-depelted cells of *N. gonorrhoeae* strain CS-7 were recorded on a Cary model 14 spectrophotometer equipped with a model 1462 scattered transmission attachment. The spectrophotometer was calibrated with a holmium oxide standard immediately prior to use. Liquid nitrogen difference spectra were recorded in a 0.2 cm-path-length cuvette attached to a low temperature tee (10). This tee was then partially immersed in liquid nitrogen contained in a glass Dewar fitted with quartz windows. Samples were reduced as described previously (38).

Miscellaneous measurements. Dry weights were obtained by centrifuging samples at 27,000 x g for 10 min to concentrate the cells. Cells were washed twice with cold distilled water and dried to constant weight in tared aluminum dishes at 80 C in a vacuum oven. Protein content of cell extracts and membrane fractions was determined by the method of Lowry *et al.* (11) with bovine serum albumin as standard. The amount of glucose remaining in the medium was determined by the glucostat method (Worthington Biochemical Corp., Freehold, N.J.). Radioactivity of samples was measured in a liquid scintillation spectrometer (Beckman Instrument Corp.) after addition of 15 ml of scintillation fluid {0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-[2]-(5-phenyloxazolyl) benzene in toluene}. The rate of protein synthesis was measured by the incorporation of [U-^{14}C] arginine into the trichloroacetic acid-insoluble cell fraction as previously described (39).

C. Results.

Acetate oxidation by glucose-grown cells. The oxidation of acetate by glucose-grown cells of N. gonorrhoeae CS-7 was determined in the presence and absence of exogenous glucose. With limiting glucose (69.4 μmol, Fig. 5), no acetate oxidation occurred until after the glucose was depleted (t = 90 min). Radiorespirometric data showed that the label in the 1 position of acetate was converted to CO_2 faster than the label in the 2 position. A similar pattern of acetate oxidation was observed 30 min following resuspension of glucose-grown cells in basal medium lacking glucose. The ratio of $[^{14}C]CO_2$ evolved from the labeled carbons of acetate (C-1/C-2) show a value consistent with the operation of a tricarboxylic acid cycle (C-1/C-2 = 2).

Tricarboxylic acid cycle and related enzymes in N. gonorrhoeae CS-7. The results (Table 2) show that glucose-grown cells contain detectable levels of all tricarboxylic acid cycle enzymes except aconitase, isocitrate dehydrogenase and a pyridine nucleotide-dependent malate dehydrogenase. All of the tricarboxylic acid cycle enzymes except the malate dehydrogenase were present in cell extracts from glucose-depleted cells. The failure to detect aconitase or isocitrate dehydrogenase was not due to the presence of a soluble inhibitor since addition of various amounts of glucose-grown cell extract to cell extract from glucose-depleted cells did not inhibit the activity of these enzymes. Aconitase and isocitrate dehydrogenase activity was not measurable following a 6-fold increase in cell extract protein. However, this experiment does not rule out the existence of these enzymes at very low levels.

A pyridine nucleotide-independent malate oxidase may be present in N. gonorrhoeae in lieu of a soluble pyridine nucleotide-dependent malate dehydrogenase. This enzyme was found in the particulate (membrane-rich) fraction from glucose-depleted cells (Table 2). Preliminary studies (Fig. 6) showed that malate reduced b and c-type cytochromes in the membrane-rich fraction of glucose-depleted cells indicating a close association of this enzyme with the electron transport chain of the gonococcus. A similar reduction of these cytochromes did not occur in membranes of glucose-grown cells unless FAD was added suggesting that this cofactor was required for enzyme activity. FAD may be absent or loosely bound to the enzyme in glucose-grown cells and therefore easily removed during preparation of the membrane-rich fraction. The peak heights seen in the difference spectra (Fig. 6) do not reflect the concentration of malic oxidase present but rather that of the cytochromes. The inability to measure malate oxidase activity in membrane preparations from glucose-grown cells by a specific enzyme assay may be due to the low specific activity of the enzyme in these cells.

Activities of enzymes catalyzing anapleurotic reactions were also measured (Table 2). Malic enzyme, PEP carboxylase, aspartate aminotransferase, and glutamic dehydrogenase(s) were present in both glucose-depleted and glucose-grown cell extracts. The presence of these enzymes in glucose-grown cells suggested that a portion of the tricarboxylic acid cycle may function in the presence of glucose. Alanine aminotransferase, citrate lyase, isocitrate lyase, and malate synthase were not detected. The absence of the latter two enzymes indicated that a glyoxylate bypass was not present in this organism under the growth conditions employed.

Effect of chloramphenicol on acetate oxidation by glucose-grown cells. The 30-min interval between the resuspension of glucose-grown cells in basal medium lacking an exogenous energy source and the rapid increase in acetate oxidation may represent the time required to derepress the synthesis of aconitase, isocitrate dehydrogenase, and malate oxidase. This possibility was examined by determining the extent of acetate oxidation by cells resuspended in basal medium with and without chloramphenicol (100 μg/ml). This concentration of chloramphenicol was chosen because it inhibited the incorporation of [U-^{14}C]arginine into the trichloroacetic acid-insoluble cell fraction by 98.5 percent. In the presence of chloramphenicol (Fig. 7), acetate was still oxidized at a slow rate suggesting that aconitase, isocitrate dehydrogenase, and malate oxidase were present. The kinetics of acetate oxidation were different with cells incubated in the presence and in the absence of chloramphenicol. A lower linear rate of [^{14}C]CO_2 evolution would be expected if acetate oxidation in the presence of chloramphenicol were the result of a decreased concentration of enzymes. However, a lower non-linear rate was observed experimentally (Fig. 7). The reasons for this are not known and are currently under investigation.

The ratio of [^{14}C]CO_2 formed from the labeled carbons of acetate (C-1/C-2) increased from 2.4 in the absence of chloramphenicol to 3.8 in its presence. This change reflects a decreased oxidation of [2-^{14}C]acetate relative to that of [1-^{14}C]acetate.

Simultaneous oxidation of acetate and glutamate. The absence of a glyoxylate bypass (Table 2) suggests that growth will be limited by the ability of the organism to remove four-carbon precursors from the tricarboxylic acid cycle for biosynthetic purposes. This need can be met by supplying compounds which can enter the TCA cycle thereby allowing another cycle intermediate to be utilized in biosynthetic reactions. The production of [^{14}C] CO_2 from labeled glutamate and acetate by cells suspended in medium containing additional glutamate (50 μmol) was determined. The addition of unlabeled glutamate was necessitated by the observation that proteose peptone no. 3 contained low levels of free glutamate. Figure 8 shows that in medium containing excess glucose (173 μmol), the label in the 1 position of glutamate was converted to CO_2 at a faster rate than the label in the 5 position. No appreciable oxidation of acetate occurred during this period. Thus, glutamate enters the tricarboxylic acid cycle during the active metabolism of glucose by N. gonorrhoeae CS-7; the absence of acetate oxidation indicates that only reactions subsequent to the formation of acetyl CoA can occur under these conditions.

Glutamate can enter the tricarboxylic acid cycle by at least two mechanisms. The low activity of glutamate aminotransferase (Table 1) suggests that this is a minor pathway. The activity of glutamic dehydrogenase(s) varied according to the pyridine nucleotide coenzyme used in the assay procedure (Table 2). Enzyme activity was greater with the coenzyme (NADP(H) than with NAD(H). The reduced form of the coenzyme was more active than the oxidized form thereby favoring the formation of glutamate from α-ketoglutarate in an in vitro system. However, the in vivo concentration of α-ketoglutarate may be sufficiently low as to shift the equilibrium towards α-ketoglutarate synthesis.

The rate of glutamate oxidation increased significantly following both a reduction in the rate of glucose metabolism, measured by the rate of [^{14}C]CO_2 production from [1-^{14}C]glucose, and a marked decrease in the rate of growth. The rate of [^{14}C]CO_2 production was equal for both labeled substrates. An increase in the rate of acetate oxidation occurred concomitantly with the decrease in glucose metabolism and growth rate. The ratio of the amount of [^{14}C]CO_2 produced from specifically labeled acetate (C-1/C-2) was approximately 2.0.

The initiation of tricarboxylic acid cycle activity was directly related to the decrease in growth rate. The production of [^{14}C]CO_2 from labeled glutamate and acetate by cells resuspended in basal medium without glucose (Fig. 9) demonstrates that in spite of concomitant oxidation of acetate and glutamate, little, if any, increase occurred in the cell yield. The increase in the rate of glutamate oxidation after 90 min was not correlated with a change in the growth rate of the organism.

The growth of N. gonorrhoeae CS-7 in basal medium supplemented with various tricarboxylic acid cycle intermediates was also examined (data not shown). Tricarboxylic acid cycle intermediates added singly (0.5% w/v final concentration) or together with acetate (0.1-0.5% w/v final concentration) failed to support the growth of strain CS-7. The entry of tricarboxylic acid cycle intermediates may require induction of a transport system. Since N. gonorrhoeae lacks endogenous metabolism (40), an external energy source may need to be supplied. This possibility was tested by inoculating N. gonorrhoeae CS-7 into basal medium containing a low concentration of glucose (0.075%) in addition to the tricarboxylic acid cycle intermediate. The amount of growth under these conditions was no greater than that in the presence of 0.075% glucose alone.

The failure of N. gonorrhoeae CS-7 to grow in medium supplemented with tricarboxylic acid cycle intermediates may be due to a lack of permeability. This possibility was examined by measuring the production of [^{14}C]CO_2 from labeled α-ketoglutarate and succinate by glucose-grown cells resuspended in basal medium without glucose. The oxidation of α-ketoglutarate and succinate by these cells (Table 3) demonstrates that the cell is permeable to at least these tricarboxylic acid cycle intermediates and that the lack of growth must be due to some other factor.

D. Discussion.

The catabolism of glucose in N. gonorrhoeae by a combination of the Entner-Doudoroff and pentose phosphate pathways (8,41) results in the accumulation of acetate in the medium (8). Studies utilizing specifically labeled [^{14}C]glucose (8) demonstrated little, if any, of the glucose carbon (C-2,C-3, C-5,C-6) excreted into the medium as acetate was further catabolized until after the glucose was exhausted from the medium. The pattern of acetate oxidation (C-1 > C-2) suggested that the tricarboxylic acid cycle was involved in the subsequent metabolism of acetate. Enzyme analyses of extracts of cells capable of oxidizing acetate confirmed the presence of all tricarboxylic acid cycle enzymes with the exception of a pyridine nucleotide-dependent malate dehydrogenase. The absence of the enzyme in N. gonorrhoeae and N. meningitidis was previously reported by Holten and Jyssum (22) and Jyssum (42) respectively.

Even though this enzyme is present in non-pathogenic *Neisseria* (22), pyridine nucleotide-dependent malate dehydrogenases are often absent in strictly aerobic bacteria. A recent report by Holten (43) suggests that gonococci contain a pyridine nucleotide-independent malate oxidase. We have confirmed this report and furthermore, have demonstrated the requirement of this enzyme for FAD and its association with the electron transport chain of *N. gonorrhoeae*. In this respect, the enzyme appears similar to those reported for *Azotobacter vinelandii* (31) and *Moraxella lwoffi* (44). However, further studies are required to firmly establish the relation of this enzyme to electron transport as well as its potential involvement in oxidative phosphorylation in *N. gonorrhoeae*.

The metabolism of glucose markedly affects both the activity of the tricarboxylic acid cycle as well as the synthesis of several tricarboxylic acid cycle enzymes. While these effects appear to be independent of one another, metabolism of glucose was required for both types of inhibition. Radiorespirometric data reflected the inhibition of acetate oxidation during the metabolism of glucose. Aconitase, isocitrate dehydrogenase, and malate oxidase were not detectable in extracts of glucose-grown cells. However, oxidation of labeled acetate in the presence of chloramphenicol by glucose-grown cells is evidence that these enzymes were present although in very low amounts. The absence of acetate oxidation during glucose metabolism, the high level of citrate synthase, and the remainder of the tricarboxylic acid cycle enzymes, point to the condensation of acetyl CoA with oxaloacetate as the key regulatory reaction. The competitive and non-competitive inhibition of citrate synthase has been reported in other organisms (45,46,47) and will be the subject of a separate paper (Hebeler and Morse, in preparation).

Fortnagel (48) reported that aconitase and isocitrate dehydrogenase in *Bacillus subtilis* were subject to feed-back repression by α-ketoglutarate or glutamate. It is unlikely that these compounds function as corepressors in *N. gonorrhoeae* as glutamate is readily converted to α-ketoglutarate and catabolized via the tricarboxylic acid cycle prior to and during the oxidation of acetate. The low specific activities of aconitase and isocitrate dehydrogenase in glucose-grown cells of *N. gonorrhoeae* may explain the failure of Tonhazy and Pelczar (21) to observe oxidation of citrate by resting cell suspensions. However, the permeability of citrate may also be an important factor (49). With the exception of citrate synthase, growth on glucose-containing medium repressed the synthesis of other tricarboxylic acid cycle enzymes. A similar repression of tricarboxylic acid cycle enzymes by glucose has been observed in other bacteria (50,51,52).

The tricarboxylic acid cycle can be divided into a series of anabolic reactions one function of which is to supply a five-carbon skeleton (α-ketoglutarate) utilized in the biosynthesis of glutamate and amino acids derived from glutamate, and a series of catabolic reactions involved in the oxidation of the same carbon skeleton. Davis (53) proposed the term "amphibolic" to describe metabolic systems, such as the tricarboxylic acid cycle, which are used for both anabolic and catabolic cellular processes. Radiorespirometric data showed that the biosynthetic portion of the tricarboxylic acid cycle, e.g., the formation of α-ketoglutarate, did not function during rapid growth of *N. gonorrhoeae* CS-7 in a peptone medium containing glucose. However, the remainder of the tricarboxylic acid cycle still functioned under these

conditions (Fig. 10). Glutamate was readily converted by the action of either a NAD- or NADP-dependent glutamate dehydrogenase (54) or a glutamate aminotransferase to α-ketoglutarate. Subsequent catabolism of α-ketoglutarate to succinyl CoA resulted in the liberation of CO_2 from the original C-1 position of glutamate. The succinate formed through the action of succinyl CoA synthetase is a symmetrical molecule which randomizes the original C-5 of glutamate equally between both carboxyl groups (C-1 and C-4) of the succinate molecule. During the rapid catabolism of an exogenous energy source such as glucose, glutamate enters the tricarboxylic acid cycle and is converted to malate. Malate thus formed, may be further catabolized by two reactions. The first, is by conversion to oxaloacetate by the enzyme malic oxidase (43). Although malic oxidase was not measurable in cell extracts from glucose-grown cells, the reduction of b and c-type cytochromes in the membrane-rich fraction and the oxidation of acetate in the presence of chloramphenicol provide indirect evidence for the existence of this enzyme at concentrations too low to be measured by the enzyme assay procedure used in this investigation. Owing to the low specific activity of malic oxidase, it was probably of minor importance in the conversion of malate to oxaloacetate during growth in glucose-containing media. The greater specific activity of the malic enzyme suggested that it was more important in the further metabolism of malate. During the conversion of malate to pyruvate, 50 percent of the original C-5 of glutamate is released as CO_2. The product, pyruvate, can be either reduced to lactate or further oxidized to acetyl CoA. In the latter reaction, the remainder of the original C-5 of glutamate will be released as CO_2. Radiorespirometric data obtained in this study are consistent with the operation of the malic enzyme.

Aspartate is a necessary precursor for protein, purine, and pyrimidine biosynthesis. Aspartate may be synthesized from fumarate by the enzyme aspartase (22) or from oxaloacetate by aspartate aminotransferase (22). Oxaloacetate is an important precursor of aspartate; a major reaction leading to its synthesis in glucose-grown cells of N. gonorrhoeae is catalyzed by PEP carboxylase. The presence of this enzyme was also reported by Holten and Jyssum (22) and was considered the major mechanism of CO_2 fixation in the gonococcus. It is not known whether the gonococcus contains pyruvate carboxylase or PEP carboxykinase but these enzymes are not found in N. meningitidis (55). Oxaloacetate is readily synthesized via malic oxidase in cells with a functional tricarboxylic acid cycle. However, unless an exogenous supply of a tricarboxylic acid cycle intermediate or some compound which can enter the cell is present the oxidation of acetate is decreased markedly due to removal of oxaloacetate through other reactions (56). A summary of the relevant tricarboxylic acid cycle activities and their relation to glucose metabolism is shown in Fig. 10.

In spite of a functional tricarboxylic acid cycle, the gonococcus cannot grow in medium supplemented with tricarboxylic acid cycle intermediates. The lack of permeability does not appear to be a factor (21,57). An alternate explanation is that the gonococcus cannot synthesize PEP from either pyruvate or tricarboxylic acid cycle intermediates. The absence of PEP carboxykinase, as previously demonstrated in N. meningitidis (55), and PEP synthetase would be sufficient to prevent the organism from growing under these conditions (58). This possibility is currently under investigation.

ADAPTATION OF THE MINITEK SYSTEM FOR THE RAPID IDENTIFICATION OF NEISSERIA GONORRHOEAE.

A. Introduction.

Both Neisseria meningitidis (60) and N. lactamica (61,62) can be isolated from the genitourinary tract on selective media commonly used for the isolation of N. gonorrhoeae (61,63). N. meningitidis can usually be differentiated from N. gonorrhoeae by the production of acid from maltose (1). However, Kingsbury (64) reported that 80% of meningococcal isolates obtained from transformation experiments and resistant to greater than 10 μg of sulfadiazine per ml were maltose negative when tested by conventional procedures, and thus, could not be differentiated from N. gonorrhoeae on the basis of sugar reactions alone. N. lactamica can be differentiated from N. meningitidis and N. gonorrhoeae by the production of acid from lactose or by the hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG) (65). In addition, many isolates of N. lactamica will grow on nutrient agar at 37 C whereas N. meningitidis and N. gonorrhoeae will not (63).

The reported incidence of gonococcal pharyngitis has increased in recent years. Its frequency varies with the particular population group studied (66,67). Hollis et al. (63) observed that 2% of a general civilian population were pharyngeal carriers of N. lactamica whereas 1.5% were pharyngeal carriers of N. meningitidis. In another study utilizing 2,224 patients at a venereal disease clinic, Wiesner et al. (67) isolated N. meningitidis from the posterior pharynx of 17.2% of the patients, N. lactamica from 1.9%, and N. gonorrhoeae from 5.6%. Thus, it becomes increasingly important to be able to accurately differentiate N. gonorrhoeae from N. meningitidis and N. lactamica.

There are several methods currently used to detect the production of acid from carbohydrates by Neisseria (68,69,70,71). A number of strains of N. gonorrhoeae have recently been encountered which either fail to produce acid from glucose or do so slowly when tested in CTA medium (65,72). The Minitek (BBL) system has recently been introduced for the identification of various bacteria. Although this system has been found particularly useful in identifying members of the Enterobacteriaceae (73), the medium supplied is unsatisfactory for determining the production of acid from various carbohydrates by N. gonorrhoeae, N. meningitidis, and N. lactamica. The purpose of this study was to develop a suspending medium which could be used with the Minitek system for the rapid differentiation of N. gonorrhoeae from N. meningitidis and N. lactamica.

B. Materials and Methods.

Organisms. Primary isolates of N. gonorrhoeae were obtained from the Venereal Disease Clinic of the Multnomah County Health Department, Portland, Oregon. Cultures were approximately 48 hours old when received and had been presumptively identified as N. gonorrhoeae by direct fluorescent antibody staining reaction and a positive oxidase test. Other clinical isolates of N. gonorrhoeae were obtained from Abdel Rashad, Clinical Microbiology Laboratory of the University of Oregon Health Sciences Center. N. gonorrhoeae

strain CS-7 has been described previously (7). Representative strains of *N. meningitidis*, with known levels of resistance to sulfadiazine, from serogroups A, B, C, X, Y, Z, 135, and 29E were obtained from Herman Schneider, Walter Reed Army Institute of Research (WRAIR), Washington, D.C. Other strains of *N. meningitidis* were obtained from the American Type Culture Collection (9). Strains of *N. lactamica* were obtained from H. Schneider, WRAIR, and from D. Kellogg, Jr., Center for Disease Control, Atlanta, Georgia. Cultures of *Neisseria* spp. were stored as previously described (7). The identity of all cultures was confirmed on the basis of appearance in gram-stained smears, oxidase reaction, and the production of acid from specific carbohydrates.

Preparation of inoculum. One-half of a plate of GC agar (Difco) containing a growth factor supplement identical in composition to IsoVitaleX enrichment (BBL) and 0.5 per cent glucose was inoculated with the test organism and incubated overnight in a CO_2 incubator (5% CO_2) at 37 C. Inocula were prepared by removing several (2-3) loopsful of cells with a 2 mm (I.D.) loop and resuspending them in 0.5 ml of medium to produce a turbid suspension containing approximately 5×10^8 colony forming units (CFU)/ml. The resuspending medium contained 1.5% (w/v) proteose peptone no. 3 (Difco) sterilized by autoclaving. Prior to use, filter-sterilized $NaHCO_3$ (4.2% w/v) was freshly added. Unless otherwise mentioned, the final concentration of $NaHCO_3$ was 210 μg/ml. In some experiments, polypeptone (BBL) and trypticase peptone (BBL) were substituted for the proteose peptone no. 3.

Minitek system. The Minitek system consists of a covered, rectangular, plastic plate containing 12 wells. The manufacturer also supplies single or multiple disc dispensers, a pipetter with sterile disposable tips, and a humidified chamber (humidor) for incubation. For use, the discs were dispensed into individual wells; three or four isolates were identified on a single Minitek plate. The discs employed in our study were dextrose + nitrate, dextrose, maltose, lactose, and ONPG. As *Neisseria* are aerobic organisms, mineral oil was not used as an overlay.

Determination of acid production from carbohydrates. Minitek discs containing glucose, maltose, sucrose, lactose, fructose, and ONPG and corresponding cystine-Trypticase agar medium (CTA, BBL) deeps containing 1% carbohydrate were inoculated with 1 drop of the inoculum suspension dispensed from the Minitek automatic pipetter. GC agar plates were simultaneously inoculated with 1 drop of inoculum and gram-stained smears prepared to monitor the initial purity of the bacterial suspensions. In later studies, inoculation of CTA medium tubes and the preparation of smears were omitted. The inoculated CTA medium tubes and Minitek plates (placed in the Minitek humidor) were incubated at 37 C without additional CO_2 and read at hourly intervals. The GC agar plates were incubated overnight at 37 C in a CO_2 incubator (5% CO_2).

Determination of inoculum size. The Minitek automatic pipetter was calibrated with an aqueous solution of [^{14}C] sodium bicarbonate (0.2 μCi/ml). Single drops dispensed by the pipetter were added to scintillation vials containing 1 ml of absolute ethanol. The vials were counted after the addition of 15 ml of scintillation fluid (0.4% 2,5-diphenyloxazole and 0.1% 1,4-bis-[2]-(5-phenyloxazolyl) benzene in toluene). Analysis of the data revealed that each drop delivered by the pipetter contained 49.66 μl $\pm$ 0.06 μl. Viable counts were determined as previously described (7).

Rapid fermentation procedure. Brown's modification (75) of the rapid fermentation procedure of Kellogg and Turner (70) was used for comparison. The procedure was as described by Brown (75) with the exception that the concentration of phenol red in the BSS diluent was doubled (W.J. Brown, personal communication). The increased phenol red concentration made the interpretation of the reactions easier. Initially, we experienced difficulty with our source of maltose. During early incubation, the phenol red would indicate an acid reaction; continued incubation often resulted in a reversion to basic pH. Brown (75) has reported a similar finding and attributed it to the contamination of maltose with low concentrations of glucose. This problem was overcome when we obtained a sample of pure maltose from W.J. Brown, CDC, Atlanta, Ga.

Chemicals and radioisotopes. The 2,5-diphenyloxazole and 1,4-bis-[2]-(5-phenyloxazolyl)-benzene were products of Fisher Scientific Co. [^{14}C] Sodium bicarbonate (specific activity 54.5 mCi/mmol) was obtained from International Chemical and Nuclear Corp. (Irvine, Calif.). All other reagents were of analytical grade.

C. Results.

Determination of optimal bicarbonate concentration. Sodium bicarbonate was added to the solution of proteose peptone no. 3 (1.5% w/v) to facilitate the differentiation of positive from negative reactions. The optimal $NaHCO_3$ concentration was determined by observing changes in the color of the phenol red indicator in the carbohydrate discs caused by *N. gonorrhoeae* strain CS-7. Table 4 shows the effects of various $NaHCO_3$ concentrations on the color changes in the indicator observed with glucose, maltose, and sucrose discs. The addition of $NaHCO_3$ decreased the time required to obtain a positive glucose reaction. At $NaHCO_3$ concentrations ranging from 26-105 μg/ml, the production of acid from glucose was ascertained by observing a change in the indicator color within 2 hours after inoculation. However, a definite negative reaction with the maltose disc was delayed. The reading of a negative reaction was made easier by increasing the sodium bicarbonate concentration to 210 μg/ml. Therefore, this concentration was used in all subsequent experiments. Concentrations of $NaHCO_3$ greater than 420 μg/ml, delayed or inhibited the appearance of a positive reaction. Substitution of polypeptone or trypticase peptone for the proteose peptone no. 3 had no appreciable effect upon a positive glucose or maltose reaction. However, the ability to discern a definite negative reaction was hampered in medium containing either polypeptone or trypticase peptone.

Effect of cell concentration. Table 5 shows that a critical cell concentration was required to effect a positive reaction within 24 hours. An increase in the cell concentration shortened this time. At very high cell densities (>5.0 x 10^9 CFU/ml), positive reactions could be read within 30 minutes. The concentration of organisms contained in 2-3 loopsful of cells resuspended in 0.5 ml of medium was determined for several strains. The results (not shown) indicated that these suspensions contained 0.5-4.0 x 10^9 CFU/ml. Therefore, with a sufficiently high concentration of cells, it should be possible to determine the carbohydrate reactions within 4 hours. The effect of incubation time was examined on 196 clinical isolates of *N. gonorrhoeae*. The results (Table 6) showed that 90.8% of the isolates produced

detectable acid from glucose within 4 hours. All isolates were identified by 6 hours. The 18 isolates which failed to give a positive reaction within 4 hours also exhibited sparse growth on GC agar plates. Twelve of these isolates were later retested at a higher cell concentration. All isolates produced acid from glucose within 3 hours and showed good growth on GC agar plates.

Strains of N. meningitidis and N. lactamica were also identified by the Minitek procedure. The results (Table 6) indicated that 97% of the N. meningitidis strains and 100% of the N. lactamica strains could be identified within 4 hours. With strains of N. meningitidis, the maltose disc always gave a positive reaction before the glucose disc. With N. lactamica, the ONPG reaction was more rapid than the production of acid from lactose.

Effect of nitrate on the production of acid from glucose by N. gonorrhoeae. The suitability of using dextrose (glucose) discs containing nitrate in place of discs containing only glucose was examined using 87 isolates of N. gonorrhoeae. The results (Fig. 11) show that the presence of nitrate in the disc markedly inhibited the production of acid from glucose by N. gonorrhoeae. With discs containing nitrate, only 56 out of 87 isolates (64.4%) gave a positive reaction within 4 hours. Eleven isolates (12.6%) failed to give a positive reaction after 24 hours of incubation. By comparison, 84 out of 87 isolates (96.6%) gave a positive reaction using glucose discs without nitrate. Only one isolate failed to give a positive reaction within 24 hours.

Validity of the Minitek system. The validity and sensitivity of the Minitek procedure for the identification of pathogenic species of Neisseria was compared with CTA medium and Brown's modification (75) of the rapid fermentation test. For the first comparison, glucose, maltose, sucrose, and lactose discs and CTA medium containing the corresponding sugars were inoculated with 1 drop (49.66 μl) of the cell suspension dispensed from the Minitek pipetter. Conditions of incubation are given in the Materials and Methods. Minitek reactions were determined within 6 hr; reaction in CTA medium were determined at 24 hr. The results of this comparison are shown in Table 7. All isolates identified using CTA medium were also identified by the Minitek system. In addition, the Minitek procedure identified more isolates of N. gonorrhoeae and N. meningitidis than were identified using CTA medium. The same two isolates of N. gonorrhoeae were not identified by either procedure; this was attributed to a low inoculum cell density. However, since both procedures used the same inoculum, it is unlikely that the 17 strains not identified by CTA medium could be attributed to low inoculum density. It was of interest that all strains of N. meningitidis tested in CTA medium produced acid from glucose; only 8 strains produced acid from maltose. The five strains which failed to produce acid from maltose were also resistant to >8.0 μg of sulfadiazine per ml. All of these strains of N. meningitidis produced acid from maltose in the Minitek system.

Table 8 shows the results of the comparison of the Minitek and rapid fermentation procedures. The same isolates were tested by each procedure. When a glucose-free maltose source was used, the rapid fermentation test gave a positive identification in less time. Both procedures correctly identified all isolates. The purity of the maltose source was very important. Two isolates were incorrectly identified as N. meningitidis because the early

acid reaction with maltose failed to revert. In addition, the early positive maltose reactions delayed the correct identification of many isolates.

D. Discussion.

The increased isolation of N. gonorrhoeae from non-genital sites as well as the isolation of N. meningitidis and N. lactamica from genital sites (60, 62,66,67,76,77) presents a problem in the clinical laboratory. All of these species of Neisseria will grow on media used for the isolation of N. gonorrhoeae (61). Therefore, isolates must be further characterized in order to ascertain the identity of the organism. The presumptive identification of an isolate as N. gonorrhoeae is based upon typical oxidase-positive colonies of a gram-negative diplococcus (78). Local policy and special situations will determine the need for a confirmatory identification of the isolate as N. gonorrhoeae. Confirmatory identification consists of either direct fluorescent antibody staining or the production of acid from specific carbohydrates. Many laboratories use both methods. Current tests for acid production from carbohydrates may give variable results due to inoculum size (75), age of inoculum, inhibitory substances in the peptone (E.S. Baron and D. Lowry, Abs. Ann. Mtng. Amer. Soc. Microbiol., 1976, p. 39), or the contamination of maltose with small amounts of glucose (75). This present study has described a rapid method for the identification of Neisseria based upon the production of acid from various carbohydrates. This method is faster and more sensitive than results obtained using CTA medium. Using identical cell concentration, the identity of 91% of the isolates could be determined with as little as 4 hours of incubation in comparison to the 24-48 hours of incubation often required with CTA medium. Furthermore, the Minitek procedure correctly identified 15% more isolates; no false positive maltose reactions were observed.

The false negative reactions occasionally observed were often due to an inoculum with a low cell density. False negative reactions have also been encountered when inocula were prepared directly from Transgrow medium or the Thayer-Martin plate used in the initial isolation of the organism. This potential problem has been overcome by re-streaking isolates on one-half of a GC agar plate. Following overnight incubation, the cells can be harvested for preparation of the inoculum. In spite of this extra step, a confirmatory identification of an isolate as N. gonorrhoeae can be made in less time than required using conventional CTA medium.

When a glucose-free maltose source is used, the rapid fermentation procedure (75) will give faster identification of N. gonorrhoeae (2 hr as compared to 4 hr). However, the difficulty in obtaining glucose-free maltose coupled with the absence of false positive maltose reactions using Minitek discs make the Minitek procedure a viable alternative method.

Glucose is metabolized in growing cells of N. gonorrhoeae by a combination of the Entner-Doudoroff and pentose phosphate pathways (8). Carbon dioxide and acetic acid are the major end products of glucose metabolism during aerobic growth (8). No growth occurs anaerobically but small amounts of acetic and lactic acids are produced from glucose (8). The presence of these acids effects a color change in the phenol red indicator contained in the Minitek discs and CTA medium thereby indicating a positive reaction.

The addition of $NaHCO_3$ to the resuspending medium affected the appearance of both positive and negative reactions. At high concentrations, HCO_3^- may act as a buffer and slow the appearance of a positive reaction. At optimal HCO_3^- concentrations, a negative reaction is easier to read as evidenced by the bright red color of the disc. In the absence of a utilizable carbohydrate, Neisseria spp. can deaminate amino acids in the proteose peptone no. 3 producing an alkaline pH (54,74). The pH may be increased further by the production of $CO_3^=$ from HCO_3^-. Other effects, such as the utilization of HCO_3^- as a growth factor (S.A. Morse and L. Bartenstein, Abs. Ann. Mtng. Amer. Soc. Microbiol., 1972, p. 44;79), cannot be eliminated at this time.

The Minitek procedure can also detect acid production from maltose by sulfadiazine resistant strains of N. meningitidis. These sulfadiazine resistant meningococci presumably lack maltose permease activity (64). However, the concentration of maltose in the Minitek discs (3 mg maltose per disc; 6% w/v final concentration) may be sufficiently high as to enter the cells by passive diffusion.

LITERATURE CITED

1. Reyn, A. 1974. Gram-negative cocci and coccibacilli. p. 427-433. In R.E. Buchanan, and N.E. Gibbons (ed.), Bergey's manual of determinative bacteriology, Williams and Wilkins, Baltimore.

2. Jurtshuk, P., and T.W. Milligan. 1974. Quantitation of the tetramethyl p-phenylene diamine oxidase reaction in Neisseria species. Appl. Microbiol. 28:1079-1081.

3. Jurtshuk, P., and T.W. Milligan. 1974. Preliminary characterization studies on the Neisseria catarrhalis respiratory electron transport chain. J. Bacteriol. 120:552-555.

4. Catlin, B.W., and L.S. Cunningham. 1961. Transforming activities and base contents of deoxyribonucleate preparations from various Neisseriae. J. Gen. Microbiol. 26:303-312.

5. Ohnishi, T. 1973. Mechanism of electron transport and energy conservation in the site I region of the respiratory chain. Biochem. Biophys. Acta 301:105-128.

6. Orme-Johnson, N.R., W.H. Orme-Johnson, R.E. Hansen, and Y. Hatefi. 1973. EPR detectable electron acceptors in sub-mitochondrial particles from beef heart. In T.E. King, H.S. Mason, and M. Morrison (ed.), Oxidases and related redox systems, University Park Press, Baltimore.

7. Morse, S.A., and L. Bartenstein. 1974. Factors affecting autolysis of Neisseria gonorrhoeae. Proc. Soc. Exp. Biol. Med. 145:1418-1421.

8. Morse, S.A., S. Stein, and J. Hines. 1974. Glucose metabolism in Neisseria gonorrhoeae. J. Bacteriol. 120:702-714.

9. Morse, S.A., and T.J. Fitzgerald. 1974. Effect of progesterone on Neisseria gonorrhoeae. Infect. Immunity 10:1370-1377.

10. Estabrook, R.W. 1961. Spectrophotometeric studies of cytochromes cooled in liquid nitrogen. p. 436-460. In J.E. Falk, R. Lemberg, and R.K. Morton (ed.), Haematin Enzymes, Perganon Press, New York.

11. Lowry, O.H., N.J. Rosenbrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

12. Beinert, H., and R.H. Sands. 1960. Studies on succinic and DPNH dehydrogenase preparations by paramagnetic resonance (EPR) spectroscopy. Biochem. Biophys. Res. Commun. 3:41-46.

13. Ohnishi, T., D.B. Winter, J. Lim, and T.E. King. 1973. Low temperature electron paramagnetic resonance studies on two iron-sulfur centers in cardiac succinate dehydrogenase. Biochem. Biophys. Res. Commun. 53:231-237.

14. Beinert, H., B.A.C. Ackrell, E.B. Kearney, and T.P. Singer. 1974. EPR studies on the mechanism of action of succinate dehydrogenase in activated preparations. Biochem. Biophys. Res. Commun. 58:564-572.

15. Lemberg, R., and J. Barrett. 1973. Cytochromes. Academic Press, Inc., New York.

16. Dietrich, W.E., and J. Biggins. 1971. Respiratory mechanisms in the Flexibacteriaceae: terminal oxidase systems of Saprospira grandis and Vitreoscilla species. J. Bacteriol. 105:1083-1089.

17. Daniel, R.M. 1970. The electron transport system of Acetobacter suboxydans with particular reference to cytochrome O. Biochim. et Biophys. Acta 216:328-341.

18. Smith, L. 1968. The respiratory chain system of bacteria. p. 55-122. In T.P. Singer (ed.), Biological oxidations, Interscience Publishers, New York.

19. Spicher, G. 1974. Cytochrome absorption spectra of bacteria as aid for solving taxonomic problems. Zbl. Bakt. Hyg. A226:524-540.

20. Broberg, P.L., and L. Smith. 1968. A comparison of the properties of the two CO-binding pigments in Bacillus megaterium KM. p. 182-187. In K. Okunuki, M.D. Kamen, and I. Sekuzu (ed.), Structure and function of cytochromes, University Park Press, Baltimore.

21. Tonhazy, N.E., and M.J. Pelczar. 1953. Oxidation of amino acids and compounds associated with the tricarboxylic acid cycle by Neisseria gonorrhoeae. J. Bacteriol. 65:368-377.

22. Holten, E. and K. Jyssum. 1974. Activities of some enzymes concerning pyruvate metabolism in Neisseria. Acta Path. Microbiol. Scandinav. 82: 843-848.

23. La Scolea, L.J., and F.E. Young. 1974. Development of a defined minimal medium for the growth of Neisseria gonorrhoeae. Appl. Microbiol. 28:70-76.

24. Okinaka, R.T., and W.J. Dobrogosz. 1967. Catabolite repression and pyruvate metabolism in Escherichia coli. J. Bacteriol. 93:1644-1650.

25. Parvin, R. 1969. Citrate synthase from yeast. p. 16-19. In J.M. Lowenstein (ed.), Methods in enzymology, vol. XIII, Academic Press, Inc, New York.

26. Fansler, B., and J.M. Lowenstein. 1969. Aconitase from pig heart, p. 26-30. In J.M. Lowenstein (ed.), Methods in enzymology, vol. XIII, Academic Press, Inc., New York.

27. Reed, L.J., and B.B. Mukherjee. 1969. α-Ketoglutarate dehydrogenase from Escherichia coli. p. 55-61. In J.M. Lowenstein (ed.), Methods in enzymology, vol. XIII, Academic Press, Inc., New York.

28. Gibson, J., C.D. Upper, and I.C. Gunsalus. 1967. Succinyl coenzyme A synthetase from Escherichia coli. I. Purification and properties. J. Biol. Chem. 242:2474-2477.

29. Johnston, K.H., and E.C. Gotschlich. 1974. Isolation and characterization of the outer membrane of Neisseria gonorrhoeae. J. Bacteriol. 119:250-257.

30. Hill, R.L., and R.A. Bradshaw. 1969. Fumarase. p. 91-99. In J.M. Lowenstein (ed.), Methods in enzymology, vol. XIII, Academic Press, New York.

31. Jurtshuk, P., A.J. Bednarz, P. Zey, and C.H. Denton. 1969. L-Malate oxidation by the electron transport fraction of Azotobacter vinelandii. J. Bacteriol. 98:1120-1127.

32. Maeba, P., and B.D. Sanwal. 1969. Phosphoenolpyruvate carboxylase from Salmonella typhimurium strain LT2. p. 283-288. In J.M. Lowenstein (ed.), Methods in enzymology, vol. XIII. Academic Press, Inc., New York.

33. Hsu, R.Y., and H.A. Lardy. 1969. Malic enzyme, p. 230-235. In J.M. Lowenstein (ed.), Methods in enzymology, vol. XIII. Academic Press, New York.

34. Jyssum, K., and B. Borchgrevink. 1960. The adaptive oxidation of l-glutamic acid in meningococci. Acta Path. Microbiol. Scandinav. 48:361-366.

35. Dixon, G.H., and H.L. Kornberg. 1962. Malate synthetase. p. 633-637. In S.P. Colowick and N.O. Kaplan (ed.), Methods in enzymology, vol. V. Academic Press, Inc., New York.

36. Carpenter, W.D., and H. Beevers. 1959. Distribution and properties of isocitratase in plants. Plant Physiol. 34:403-409.

37. Dagley, S. 1969. Citrate lyase. p. 160-163. In J.M. Lowenstein (ed.), Methods in enzymology, vol. XIII. Academic Press, Inc., New York.

38. Winter, D.E., and S.A. Morse. 1975. Physiology and metabolism of pathogenic Neisseria: Partial characterization of the respiratory chain of Neisseria gonorrhoeae. J. Bacteriol. 123:631-636.

39. Morse, S.A., R.A. Mah, and W.J. Dobrogosz. 1969. Regulation of staphylococcal enterotoxin. B.J. Bacteriol. 98:4-9.

40. Barron, E.S.G., and C.P. Miller, Jr. 1932. Studies on biological oxidations. I. Oxidations produced by gonococci. J. Biol. Chem. 97:691-715.

41. Holten, E. 1975. Radiorespirometric studies in genus Neisseria. I. The catabolism of glucose. Acta Path. Microbiol. Scandinav. 83:353-366.

42. Jyssum, K. 1960. Intermediate reactions of the tricarboxylic acid cycle in meningococci. Acta Path. Microbiol. Scandinav. 48:121-132.

43. Holten, E. 1976. Pyridine nucleotide independent oxidation of l-malate in genus Neisseria. Acta Path. Microbiol. Scandinav. 84:17-21.

44. Jones, M., and H.K. King. 1972. Particulate malate oxidation in strictly aerobic bacteria: The respiratory chain of Moraxella lwoffi. FEBS Lett. 22:277-279.

45. Fletchner, V.R., and R.S. Hanson. 1970. Regulation of the tricarboxylic acid cycle in bacteria. A comparison of citrate synthase from different bacteria. Biochim. Biophys. Acta 222:253-264.

46. Weitzman, P.D.J., and P. Dunmore. 1969. Citrate synthase: Allosteric regulation and molecular size. Biochim. Biophys. Acta 171:198-200.

47. Weitzman, P.D.J., and D. Jones. 1975. The mode of regulation of bacterial citrate synthase as a taxonomic tool. J. Gen. Microbiol. 89:187-190.

48. Fortnagel, P. 1970. The regulation of aconitase and isocitrate dehydrogenase in sporulation mutants of Bacillus subtilis. Biochim. Biophys. Acta 222: 290-298.

49. Hill, J.C. 1971. Effect of glutamate on exogenous citrate catabolism of Neisseria meningitidis and of other species of Neisseria. J. Bacteriol. 106:819-823.

50. Cho, H.W., and R.G. Eagon. 1967. Factors affecting the pathways of glucose catabolism and the tricarboxylic acid cycle in Pseudomonas natriegens. J. Bacteriol. 93:866-873.

51. Gray, C.T., J.W.T. Wimpenny, and M.R. Mossman. 1966. Regulation of metabolism in facultative bacteria. II. Effects of aerobiosis, anaerobiosis and nutrition on the formation of Krebs cycle enzymes in Escherichia coli. Biochem. Biophys. Res. Commun. 59:1204-1210.

52. Hanson, R.S., and D.P. Cox. 1967. Effect of different nutritional conditions on the synthesis of tricarboxylic acid cycle enzymes. J. Bacteriol. 93:1777-1787.

53. Davis, B.D. 1961. The teleonomic significance of biosynthetic control mechanisms. Cold Spring Harbor Symp. Quant. Biol. 26:1-10.

54. Holten, E. 1973. Glutamate dehydrogenases in genus Neisseria. Acta Path. Microbiol. Scandinav. 81:49-58.

55. Jyssum, K., and S. Jyssum. 1962. Phosphoenolpyruvic carboxylase activity in extracts from Neisseria meningitidis. Acta Path. Microbiol. Scandinav. 54:412-424.

56. Holten, E. 1976. Radiorespirometric studies in genus Neisseria. 3. The catabolism of pyruvate and acetate. Acta Path. Microbiol. Scandinav. 84: 9-16.

57. Holten, E. 1976. Radiorespirometric studies in genus Neisseria. 2. The catabolism of glutamate and fumarate. Acta Path. Microbiol. Scandinav. 84:1-8.

58. Hansen, E.J., and E. Juni. 1974. Two routes for synthesis of phosphoenolpyruvate from C_4-dicarboxylic acids in Escherichia coli. Biochem. Biophys. Res. Commun. 59:1204-1210.

59. Kellogg, D.S., Jr., W.L. Peacock, Jr., W.E. Deacon, L. Brown, and C.I. Pirkle. 1963. Neisseria gonorrhoeae. I. Virulence genetically linked to colonial variation. J. Bacteriol. 85:1274-1279.

60. Lewis, J.F., and J.J. Alexander. 1974. Isolation of Neisseria meningitidis from the vagina and cervix. Am. J. Clin. Path. 61:216-217.

61. Edberg, S. 1974. The growth of Neisseria lactamica on media selective for pathogenic Neisseriaceae. Am. J. Clin. Path. 62:445.

62. Jephcott, A.E., and R.S. Morton. 1972. Isolation of Neisseria lactamicus from a genital site. Lancet 2:739-740.

63. Hollis, D.G., G.L. Wiggins, R.E. Weaver, and J.H. Schubert. 1970. Current status of lactose fermenting Neisseria. Ann. N.Y. Acad. Sci. 174:444-449.

64. Kingsbury, D.T. 1967. Relationship between sulfadiazine resistance and the failure to ferment maltose in Neisseria meningitidis. J. Bacteriol. 94:557-561.

65. Catlin, B.W. 1973. Nutritional profiles of Neisseria gonorrhoeae, Neisseria meningitidis, and Neisseria lactamica in chemically defined media and the use of growth requirements for gonococcal typing. J. Infect. Dis. 128: 178-194.

66. Corman, L.C., M.E. Levison, R. Knight, E.R. Carrington, and D. Kaye. 1974. The high frequency of pharyngeal gonococcal infection in a prenatal clinic population. J. Amer. Med. Assoc. 230:568-570.

67. Wiesner, P.J., E. Tronca, P. Bonin, A.H.B. Pedersen, and K.K. Holmes. 1973. Clinical spectrum of pharyngeal gonococcal infection. N. Eng. J. Med. 288: 181-185.

68. Bailey, W.R., and E.G. Scott. 1974. Diagnostic microbiology, 4th Ed., C.V. Mosby Co., St. Louis.

69. Davies, J.A., J.R. Mitzell, and W.E. Beam, Jr. 1971. Carbohydrate fermentation patterns of Neisseria meningitidis determined by a microtiter method. Appl. Microbiol. 21:1072-1074.

70. Kellogg, D.S., Jr., and E.M. Turner. 1973. Rapid fermentation confirmation of Neisseria gonorrhoeae. Appl. Microbiol. 25:550-552.

71. Stacey, P., and G. Warner. 1973. Identification of Neisseria gonorrhoeae and Neisseria meningitidis by a carbohydrate disc technique. J. Clin. Path. 26:303-304.

72. White, L.A., and D.S. Kellogg, Jr. 1965. An improved fermentation medium for Neisseria gonorrhoeae and other Neisseria. Health Lab. Sci. 2:238-241.

73. Kiehn, T.E., K. Brennan, and P.D. Ellner. 1974. Evaluation of the Minitek system for identification of Enterobacteriaceae. Appl. Microbiol. 28: 668-671.

74. Holten, E., and K. Jyssum. 1973. Glutamate dehydrogenases in Neisseria meningitidis. Acta Path. Microbiol. Scand. 81:43-48.

75. Brown, W.J. 1974. Modification of the rapid fermentation test for Neisseria gonorrhoeae. Appl. Microbiol. 27:1027-1030.

76. Beck, A., J.L. Fluker, and D.J. Platt. 1974. Neisseria meningitidis in urogenital infection. Brit. J. Vener. Dis. 50:367-369.

77. Givan, K.F., and A. Keyl. 1974. The isolation of Neisseria species from unusual sites. Canadian Med. Assoc. J. 111:1077-1079.

78. Balows, A., and D.W. Printz. 1972. CDC program for diagnosis of gonorrhea. Letters. J. Amer. Med. Assoc. 222:1557.

79. Talley, R.S., and C.L. Baugh. 1975. Effects of bicarbonate on growth of Neisseria gonorrhoeae: Replacement of gaseious CO_2 atmosphere. Appl. Microbiol. 29:469-471.

Table 1. Cytochrome concentration in membrane-fragments from N. gonorrhoeae CS-7.

Cytochrome[a]	Concentration (nmoles/mg protein)
cytochrome c	1.15
cytochrome b_{557}	0.75
cytochrome b_{562}	0.13
cytochrome o	0.18

[a] The designation cytochrome is used here for convenience and is based only upon absorbance peak positions in comparison with established absorbance maxima observed in other bacterial and mitochondrial fragments. No information as to the structure of the heme prosthetic group is intended by this designation.

Table 2. Activities of tricarboxylic acid cycle and related enzymes in cell extracts from glucose-grown and glucose-depleted cells of *N. gonorrhoeae* CS-7.[a]

Enzyme	Specific Activity[b] Glucose-grown	Glucose-depleted
Citrate synthase	1612	400
Aconitase	ND[c]	5.9
Isocitrate dehydrogenase	ND	46
α-Ketoglutarate dehydrogenase complex	0.13	2.1
Succinyl CoA synthetase	0.24	0.43
Succinic dehydrogenase	18.1	101.5
Fumarase	1.5	3.8
Malate dehydrogenase	ND	ND
Malate oxidase	ND	0.7
PEP carboxylase	0.4	ND
Malic enzyme	4.1	1.1
Citrate lyase	ND	ND
Isocitrate lyase	ND	ND
Malate synthase	ND	ND
Aspartate aminotransferase	0.01	0.02
Alanine aminotransferase	ND	ND
Glutamic dehydrogenase (NAD)[d]	0.42	3.4
Glutamic dehydrogenase (NADH)[d]	5.8	4.8
Glutamic dehydrogenase (NADP)[d]	215	95.4
Glutamic dehydrogenase (NADPH)[d]	739	121

a. Assay procedures are as described.

b. All activities are expressed as μmoles per minute per milligram of protein.

c. ND = none detected.

d. Coenzyme used in assay procedure.

Table 3. Utilization of labeled α-ketoglutarate and succinate by *N. gonorrhoeae* CS-7.[a]

Substrate	Respiratory CO_2 (%)	Radioactive inventory[b] Cells (%)	Medium (%)	Total ^{14}C recovery (%)
$[1-^{14}C]$α-ketoglutarate	51.2	1.3	47.5	96.7
$[1,4-^{14}C]$succinate	18.4	1.7	79.8	100

[a] Flasks contained 25 mg (dry weight) of cells, 100 μmol of sodium acetate, 50 μmol of monosodium glutamate, and 50 μmol of either potassium α-ketoglutarate or sodium succinate. The activity of the labeled compounds was approximately 1.0 μCi/ml.

[b] Determined after 120 min of incubation.

Table 4. Effect of sodium bicarbonate concentration on Minitek reactions.[a]

Hours	Carbohydrate disc	Sodium bicarbonate concentration ($\mu g/ml$) 420	210	105	52.5	26.2	13.1	6.6
1	Glucose	R/O	R/O	R/O	R/O	R/O	R/O	R/O
	Maltose	R	R	R/O	R/O	R/O	R/O	R/O
	Sucrose	R	R	R	R	R	R	R
2	Glucose	O	O	Y/O	Y/O	Y/O	O	O
	Maltose	R	R	R/O	R/O	R/O	R/O	R/O
	Sucrose	R	R	R	R	R	R	R
3	Glucose	Y/O	Y/O	Y	Y	Y	Y/O	Y/O
	Maltose	R	R	R	R/O	R	R/O	R
	Sucrose	R	R	R	R	R	R	R
4	Glucose	Y	Y	Y	Y	Y	Y/O	Y/O
	Maltose	R	R	R	R	R	R	R
	Sucrose	R	R	R	R	R	R	R
5	Glucose	Y	Y	Y	Y	Y	Y	Y
	Maltose	R	R	R	R	R	R	R
	Sucrose	R	R	R	R	R	R	R

[a] Y = yellow (positive reaction); Y/O = yello-orange (positive reaction; O = orange (intermediate reaction); R/O = red-orange (negative reaction); R = red (negative reaction).

Table 5. Effect of cell concentration on production of acid from glucose by *N. gonorrhoeae* CS-7.[a]

	Cell concentration (CFU/ml / CFU/well)							
	4.7×10^{9}	2.3×10^{9}	1.2×10^{9}	5.9×10^{8}	2.9×10^{8}	1.5×10^{8}	7.3×10^{7}	3.7×10^{7}
Hours	2.4×10^{8}	1.2×10^{8}	5.9×10^{7}	2.9×10^{7}	1.5×10^{7}	7.3×10^{6}	3.7×10^{6}	1.8×10^{6}
1	Y/O	O	R/O	R/O	R	R	R	R
2	Y	Y/O	Y/O	O	R/O	R	R	R
3	Y	Y	Y	Y/O	O	R/O	R/O	R
4	Y	Y	Y	Y/O	O	O	R/O	R/O
5	Y	Y	Y	Y	O	O	R/O	R/O
6	Y	Y	Y	Y	Y/O	O	O	R/O
11	Y	Y	Y	Y	Y/O	Y/O	O	O
24	Y	Y	Y	Y	Y	Y/O	O	O

[a] Y = yellow (positive reaction); Y/O = yellow orange (positive reaction); O = orange (intermediate reaction); R/O = red-orange (negative reaction); R = red (negative reaction).

Table 6. Effect of incubation time on the identification of *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica*.

Organism	Number of strains tested	Cumulative number of strains showing positive reactions at 1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
N. gonorrhoeae	196	38	117	166	178	191	196
	(%)	(19.4%)	(59.7%)	(84.7%)	(90.8%)	(97.4%)	(100%)
	12[a]	5	11	12	-	-	-
	(%)	(41.7%)	(91.7%)	(100%)			
N. meningitidis[b]	32	16	25	29	31	31	32
	(%)	(50.0%)	(78.1%)	(90.5%)	(96.7%)	(96.7%)	(100%)
N. lactamica[c]	8	2	8	-	-	-	-
	(%)	(25.0%)	(100%)				

[a] Strains which failed to produce acid from glucose within 4 hours.

[b] Identification as *N. meningitidis* requires positive glucose and maltose reactions.

[c] Identification as *N. lactamica* requires positive glucose, maltose, and lactose reactions.

Table 7. Comparison of Minitek and CTA procedures.

Organism	Number of strains examined	Number of strains identified by	
		Minitek	CTA
N. gonorrhoeae	110	108 (98 %)	91 (82.7%)
N. meningitidis	13	13 (100%)	8 (61.4%)[a]
N. lactamica	8	8 (100%)	8 (100 %)

[a] No strains resistant to $\geq$0.8 μg of sulfadiazine per ml were identified by the CTA procedure.

Table 8. Comparison of Minitek and rapid fermentation procedures for identification of *N. gonorrhoeae*.

Method	Number of strains tested	Cumulative number of strains showing positive reactions at 1 hr	2 hr	3 hr	4 hr
Minitek	20 (%)	2 (10%)	12 (60%)	19 (95%)	20 (100%)
Rapid fermentation[a]	20	18 (90%)	20 (100%)		
Rapid fermentation[b]	20	9 (45%)	18 (90%)	18 (90%)	18[c] (90%)

[a] Sugars obtained from W. J. Brown, CDC, Atlanta, Ga.

[b] Sugars obtained from commercial sources.

[c] Two isolates identified as *N. meningitidis* on the basis of positive glucose and maltose reactions.

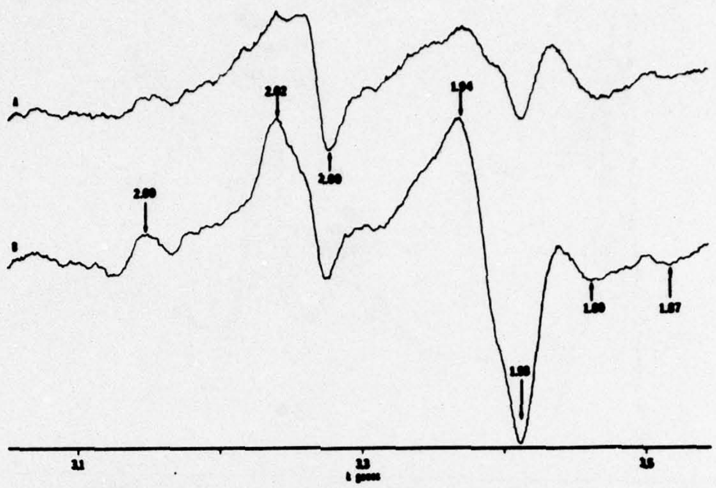

FIG. 1. *Low-temperature EPR spectra of reduced Neisseria gonorrhoeae cell membranes. N. gonorrhoeae membranes (50 mg/ml) dissolved in 0.4 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.005 M mercaptoethanol (pH 7.4) were made anaerobic under argon and reduced with a small amount of solid sodium succinate (A) or dithionite (B). The EPR operating conditions were: modulation amplitude, 9.7 gauss; microwave power, 25 mW; microwave frequency, 9.202 GHz; time constant, 1.0 s; scanning rate, 100 gauss per min; temperature, 18.0 K.*

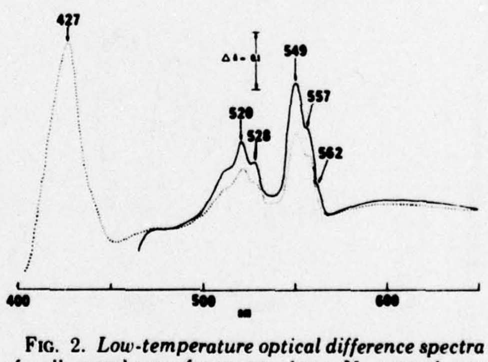

FIG. 2. *Low-temperature optical difference spectra of cell membrane fragments from N. gonorrhoeae CS-7. Dithionite-reduced minus ferricyanide-oxidized (solid line) and dithionite-reduced minus air-oxidized (dotted line) spectra of N. gonorrhoeae cell membrane fragments [10 mg of protein/ml in 0.08 M tris(hydroxymethyl)aminomethane-hydrochloride, 10^{-3} M β-mercaptoethanol, pH 7.4]. Optical difference spectra were recorded as described in Materials and Methods. Samples were reduced under anaerobic conditions with solid sodium dithionite. Ferricyanide (100 μM) was added to the reference sample.*

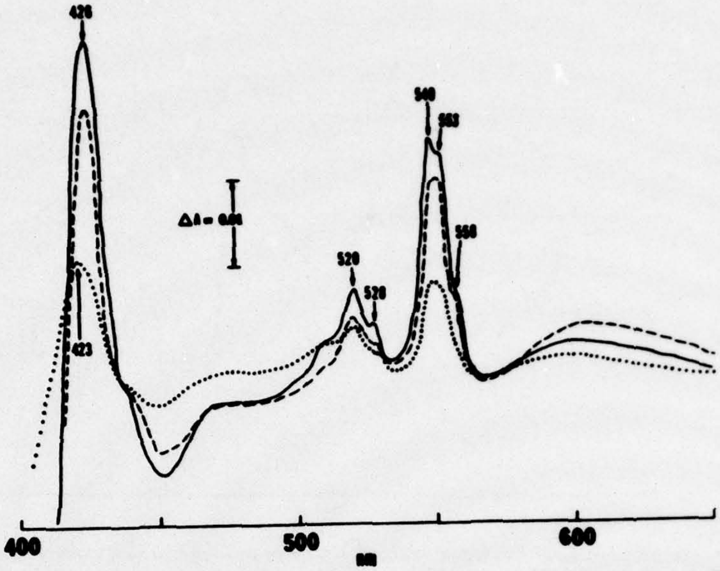

FIG. 3. *Low-temperature optical difference spectra of succinate-, L-lactate-, and ascorbate-reduced N. gonorrhoeae cell membranes. Solid sodium succinate (dashed line), sodium L-lactate (solid line), and sodium L-ascorbate (dotted line) were added to oxygen-free N. gonorrhoeae membrane fragments (10 mg of protein/ml). Optical difference spectra were recorded as described in Materials and Methods.*

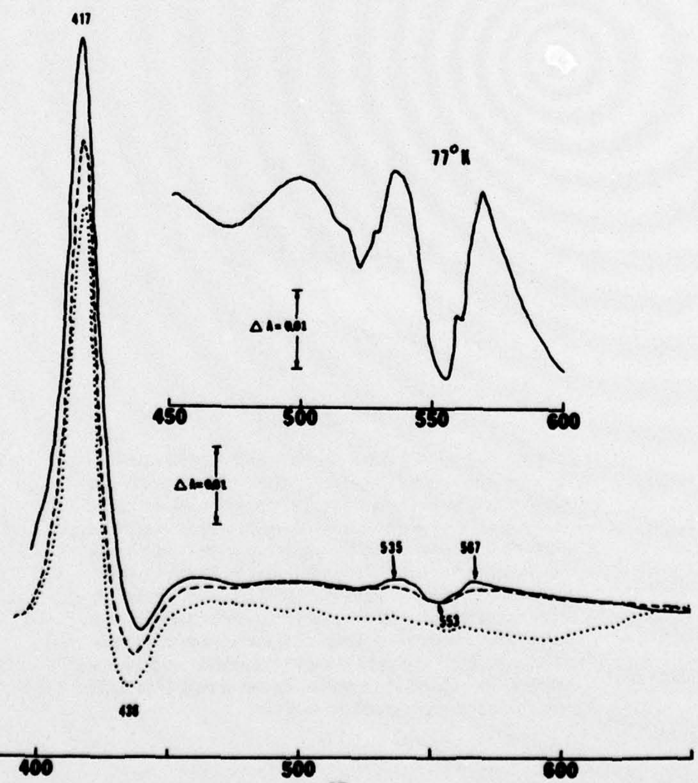

FIG. 4. *Room temperature carbon monoxide difference spectra of N. gonorrhoeae. Dithionite-reduced N. gonorrhoeae cell-membrane particles (12.6 mg of protein/ml) were bubbled with CO for 90 s (dotted line), 5 min (dashed line), and 15 min (solid line), and optical difference spectra were recorded. Inset is the 77 K spectra of the particles bubbled with CO for 15 min.*

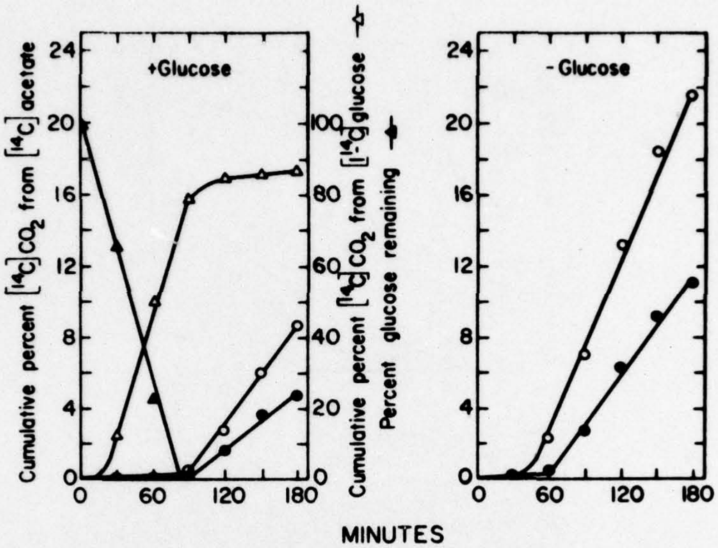

Fig. 5. *Radiorespirometric patterns for the utilization of labeled acetate by glucose-grown N. gonorrhoeae CS-7. Flasks contained 25 mg (dry weight) of cells, 100 µmol of sodium acetate and, where indicated, 69.4 µmol of glucose. The activity of the labeled compounds was approximately 1.0 µCi/ml. Symbols: (○) [1-¹⁴C]-acetate, (●) [2-¹⁴C]acetate. Results are expressed as (activity of [¹⁴C]CO_2/activity of ¹⁴C-labeled substrate added) × 100%.*

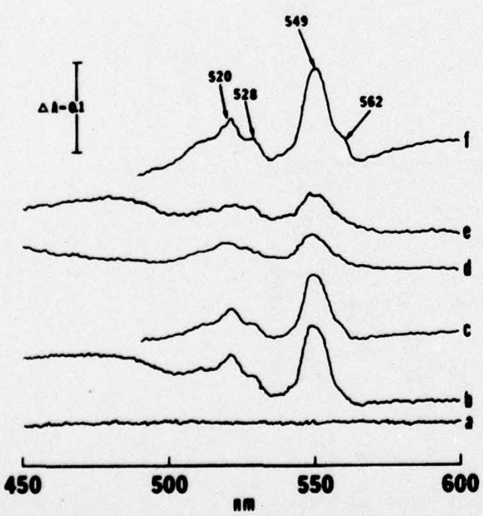

Fig. 6. *Low-temperature optical difference spectra of L-malate-reduced membrane fragments from glucose-grown (a, b, c) and glucose-depleted (d, e, f) cells of N. gonorrhoeae CS-7. (a) and (d) L-Malate-reduced minus air-oxidized spectra. (b) and (e) FAD plus L-malate-reduced minus air-oxidized spectra. (c) and (f) FAD plus L-malate-reduced minus ferricyanide-oxidized spectra. Optical difference spectra were recorded as described in Materials and Methods. Samples were reduced under anaerobic conditions with solid sodium L-malate either in the presence or absence of exogenous FAD (35 µM). Ferricyanide (100 µM) was added to the reference samples as indicated.*

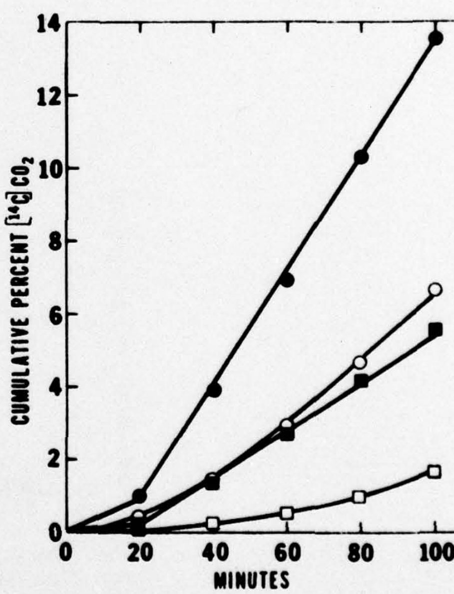

Fig. 7. *Effect of chloramphenicol on acetate oxidation by glucose-grown cells of N. gonorrhoeae CS-7. Glucose-grown cells were resuspended in basal medium with and without chloramphenicol (100 µg/ml). Flasks contained 25 mg (dry weight) of cells and 100 µmol of sodium acetate. The activity of the labeled acetate was approximately 1.0 µCi/ml. Symbols: (○) [1-¹⁴C]acetate plus chloramphenicol, (□) [2-¹⁴C]acetate plus chloramphenicol, (●) [1-¹⁴C]acetate, and (■) [2-¹⁴C]acetate. Results are expressed as (activity of [¹⁴C]CO_2/activity of ¹⁴C-labeled substrate added) × 100%.*

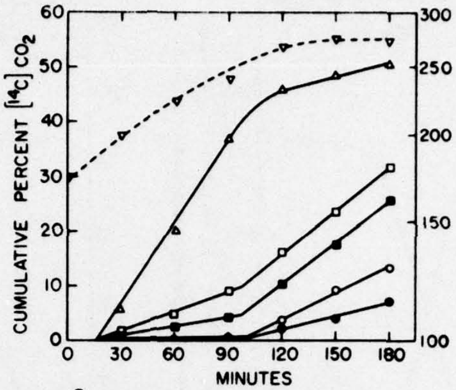

FIG. 8. *Radiorespirometric patterns for the utilization of labeled acetate and glutamate during growth on glucose by N. gonorrhoeae CS-7. Flasks contained 25 mg (dry weight) of cells, 100 μmol of sodium acetate, 173 μmol of glucose, and 50 μmol of exogenous monosodium glutamate. The activity of the labeled compounds was approximately 1.0 μCi/ml. Symbols: (○) [1-¹⁴C]acetate, (●) [2-¹⁴C]acetate, (□) [1-¹⁴C]glutamate, (■) [5-¹⁴C]glutamate, (△) [1-¹⁴C]glucose, and (▽) turbidity. Results are expressed as (activity of [¹⁴C]CO₂/activity of ¹⁴C-labeled substrate added) × 100%.*

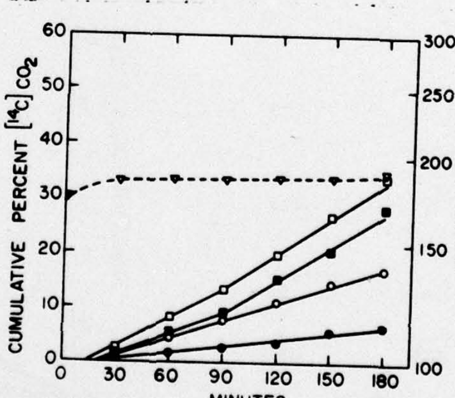

FIG. 9. *Radiorespirometric patterns for the utilization of labeled acetate and glutamate during the incubation of N. gonorrhoeae CS-7 in basal medium lacking glucose. Flasks contained 25 mg (dry weight) of cells and 100 μmol of exogenous monosodium glutamate. The activity of the labeled compounds was approximately 1.0 μCi/ml. Symbols: (○) [1-¹⁴C]acetate, (●) [2-¹⁴C]acetate, (□) [1-¹⁴C]glutamate, (■) [5-¹⁴C]glutamate, (▽) turbidity. Results are expressed as (activity of [¹⁴C]CO₂/activity of ¹⁴C-labeled substrate added) × 100%.*

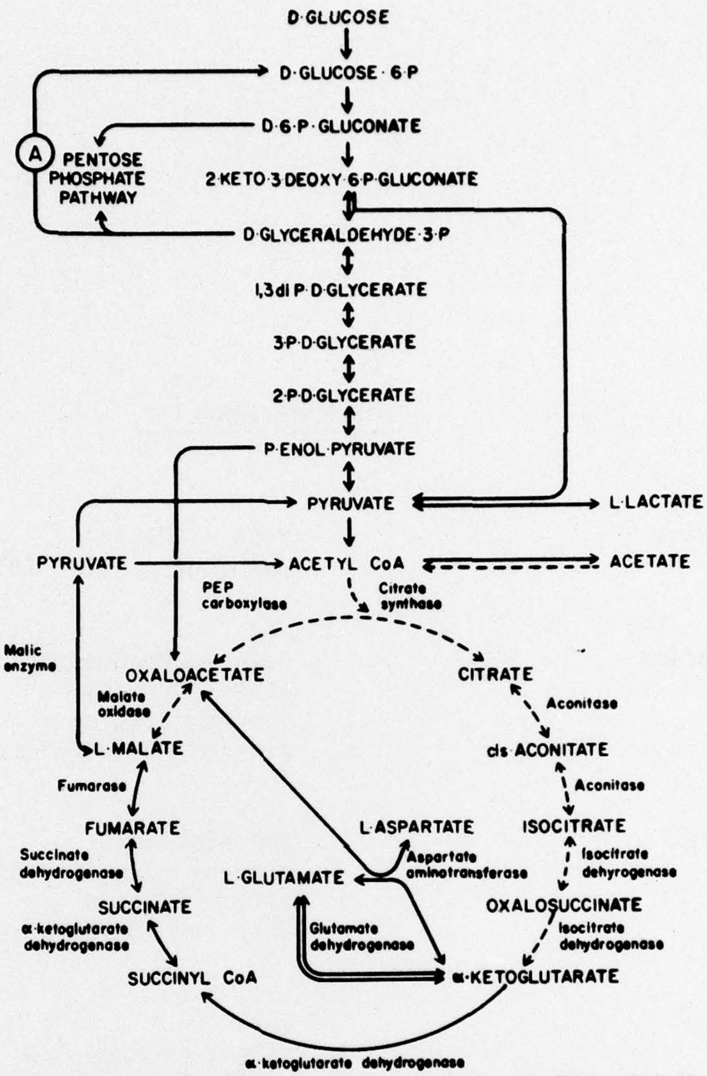

FIG. 10. *Pathway for the catabolism of glucose and the involvement of the TCA cycle in N. gonorrhoeae CS-7. The reactions indicated by the symbol (A) are given in detail in reference (36). Reactions indicated by solid arrows occur during growth on glucose; those indicated by the dashed arrows occur after glucose depletion or after the cessation of exponential growth.*

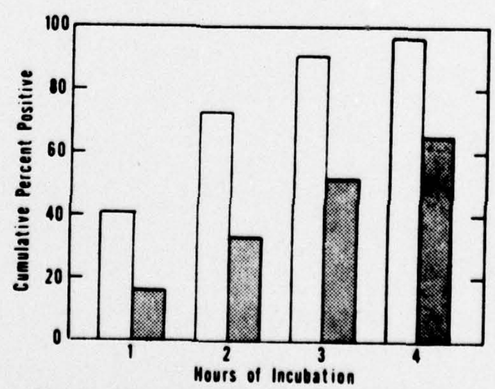

FIG. 11. *Effect of nitrate on the production of acid from glucose by clinical isolates of Neisseria gonorrhoeae. Open bars, Glucose disks without nitrate; stippled bars, glucose disks containing nitrate.*

DISTRIBUTION LIST

4 copies	HQDA (SGRD-AJ) Washington, D.C. 20314
12 copies	Defense Documentation Center (DDC) ATTN: DDC-TCA Cameron Station Alexandria, Virginia 22314
1 copy	Superintendent Academy of Health Sciences, US Army ATTN: AHS-COM Fort Sam Houston, Texas 78234
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences Office of the Secretary of Defense 6917 Arlington Road Bethesda, Maryland 20014